Contributions of the Human SSB Complex and MEI5-SWI5 Complex to Homologous Recombination

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CONTRIBUTIONS OF THE HUMAN SSB COMPLEX AND MEI5-SW15 COMPLEX TO HOMOLOGOUS RECOMBINATION

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biochemistry and Molecular Biology

by
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December 2014

Accepted by:
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ABSTRACT

DNA double-strand breaks (DSBs) are the most threatening type of DNA damage in a cell. Homologous recombination (HR) is the most accurate repair mechanism for DSBs, and if HR fails, the integrity of the genome can be compromised. Two recombinases, RAD51 and DMC1, are vital for HR but require assistance for HR to proceed efficiently and accurately. Several proteins, including mediators, single-strand binding proteins, and accessory proteins, have been shown to function in the HR with the recombinases. Mediators are responsible for overcoming inhibition caused by the single-strand binding protein, Replication protein A (RPA). Accessory proteins assist the recombinases through DSB localization, ATP hydrolysis, filament stabilization and several other functions.

In addition to RPA, higher eukaryotes possess two other SSBs, SSB1 and SSB2. Both hSSBs maintain genomic integrity through participation in the HR pathway. It was previously demonstrated that hSSB1 stimulates RAD51 during D-loop formation. Additionally, the hSSBs maintain genomic integrity through the repair of stalled replication forks. In this dissertation, we present in Chapter 2 surprising activities of the hSSBs that support the recent genetic data implicating hSSB1 and hSSB2 in the repair of stalled replication forks. We demonstrated a functional interaction with the human polymerase η in D-loop extension and second-end capture. This is the first report of the hSSBs interaction with a polymerase and identifies a new function of the hSSBs in DNA double-strand break repair. We also report that hSSB1 and hSSB2 can anneal single-strand DNA and melt double-strand DNA.
In Chapter 3, we examined the effect of CaCl\(_2\) and MgCl\(_2\) on hSSB D-loop formation and demonstrate that hSSB1 and hSSB2 can in fact form D-loops in the absence of the recombinase, RAD51. Both hSSB1 and hSSB2 form a heterotrimeric complex with Integrator subunit 3 (INTS3) and the Single-strand interacting protein 1 (hSSBIP1). We have purified the components and confirmed complex formation. The effect of the complex proteins on D-loop extension by hPol \(\eta\) will be interesting to examine in the future.

The hMEI5-SWI5 ortholog in *Saccharomyces cerevisiae* functions as a mediator to scDMC1. To date, there have been no reports regarding hMEI5-SWI5 functionality with hDMC1. In Chapter 3, we examined the DNA binding activity of hMEI5 and hSWI5 individually and as a complex (Mei5-Swi5), in addition to demonstrating physical interaction with both DMC1 and RPA. Importantly, we report that hMEI5 but not hSWI5 retains mediator activity to hDMC1 using an *in vitro* homologous DNA pairing assay. This is the first biochemical report on hMEI5-hSWI5.
DEDICATION

This dissertation is dedicated to my daughter, Haley Ledford.
ACKNOWLEDGEMENTS

First, I would like to take this opportunity to thank my daughter, mother and father for their understanding and support throughout my time at Clemson University. Over the years, they have both been a constant source of love and inspiration. Additionally, I would like to thank Tammy Stewart and Dru Brown, for their help and encouragement during these last few months.

I would also like to thank all of the members of the Sehorn lab for their encouragement and motivation throughout the years, especially Dr. Deepti Sharma, Dr. Amanda Say, Shivani Shah and Drew Kelso.
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CHAPTER 1
LITERATURE REVIEW

Introduction

DNA damage can compromise the integrity of the genome if left unrepaired. DNA double-strand (DSB) breaks are the most deleterious type of damage that can occur, and it is estimated ~ 10 DSBs occur daily in each cell. (Lieber et al., 2010). Exogenous DSBs can arise from exposure to ionizing radiation or free radicals. Programmed endogenous DSBs (during meiosis or V(D)J recombination) are beneficial and provide a mechanism to increase genetic diversity. However, spontaneous endogenous DSBs that occur from replication of a damaged DNA template or fork collapse can be detrimental if not repaired correctly (Keeney and Neale 2006, Grawunder et al., 1998a). If a single DSB is left unrepaired, severe consequences such as chromosomal aneuploidy, translocations or even cell death may occur (Rudin and Haber, 1988, Carney et al., 1998, Lim and Hasty, 1996). Furthermore, a breakdown in the DSB repair pathway can lead to carcinogenesis, birth defects or other diseases such as Fanconi Anemia (FA) (Nakanishi et al., 2005, Ghosal and Chen, 2013).

There are several pathways to repair DSBs, including non-homologous end joining (NHEJ) and homologous recombination (HR). Although some overlap in the NHEJ and HR is present, each pathway requires specific proteins to proceed efficiently. The DSB repair pathways and a subset of the proteins involved in each mechanism will be reviewed.
**DSB Repair Pathways**

There are two main DSB repair pathways – non-homologous end joining (NHEJ) and homologous recombination (HR). Although NHEJ is error-prone, it is necessary to repair DSBs when a homologous chromatid is not available to serve as a template as well as during V(D)J recombination (Moore and Haber, 1996, Malu et al., 2012). HR is partly responsible for generating genetic diversity while maintaining genomic integrity and is considered to be predominately error-free (Haber, 1998, Krogh and Symington, 2004). HR is therefore the preferred DSB repair mechanism. However, HR predominately functions during the S or G2 cell cycle phase while NHEJ is active throughout the cell cycle (Takashima et al., 2009).

**Non-homologous End Joining (NHEJ)**

The first step in non-homologous end joining (NHEJ) is end-processing and complex formation by the heterodimer Ku70/Ku80 (Figure 1.1). Ku70/Ku80 recognizes the DSB and serves as a scaffold protein for the DNA-dependent protein kinase (DNA-PKcs) (Uematsu et al., 2007). Bridging of the DNA ends occurs after DNA-PKcs binds to the DNA, which is then processed by the enzyme Artemis to produce short single-strand regions (Ma et al., 2002). A complex composed of DNA ligase IV and the X-ray repair complementing group 4 (XRCC4) ligates the microhomologous regions before the remaining gaps are filled in by polymerases (Wilson, et al., 1997, Grawunder et al., 1998b, Drouet et al., 2005). However, if the short homologous DNA regions are compatible and free 5' phosphate and 3'OH ends are available, genomic integrity can
become compromised. Accordingly, NHEJ is often associated with chromosomal translocations (Yu and Gabriel, 2004).

**Figure 1.1 Mechanism of Non-homologous end joining (NHEJ).** The heterodimer Ku70/Ku80 (brown and orange) senses the DSB and initiates the NHEJ pathway. DNA-PKcs (purple) binds the dsDNA before end processing by the enzyme Artemis (yellow) to produce short single-strand regions that are ligated by a complex composed of DNA ligase IV and X-ray repair complementing group 4 (XRCC4) (pink). Adapted with permission from Peng and Lin, 2011.

**Homologous Recombination**

Homologous recombination (HR) is typically an error-free mechanism for the repair of DSBs, maintaining genomic integrity and generating genetic diversity during meiosis (Keeney and Neale, 2006, Krogh and Symington, 2004). These important functions are accomplished by the action of two *E. coli* RecA-like recombinase proteins
in eukaryotes, RAD51 and DMC1. Both recombinases are assisted by mediators and accessory factors to increase the efficiency of HR.

Once a DSB is introduced either exogenously or endogenously in somatic cells, the MRN complex (Mre11, Rad50 and Nbs1) recognizes the break and activates the transducer kinases ATM (ataxia telangiectasia-mutated) or ATR (ataxia telangiectasia-mutated and Rad3-related) to initiate a phosphorylation signaling cascade. This signaling cascade leads to cell cycle arrest (Petrini, 2000, Kastan and Lim, 2000). In meiosis, programmed DSBs are created by the topoisomerase, Spo11 (Keeney et al., 1997). The 5' ends of the dsDNA breaks are then nucleolytically resected by exonucleases and the MRN complex to produce 3' overhang regions of ssDNA. CtIP also assists the MRN complex to initiate resection before the exonuclease Exo1 resects the DNA further (Figure 1.2).

The heterotrimeric ssDNA binding protein Replication Protein A (RPA) coats the 3' ssDNA to prevent reannealing and formation of secondary structure (Sugiyama et al., 1997, Sung, 1997a). Mediators such as Rad52 and BRCA2 are required to remove RPA from the ssDNA before the recombinase can bind and form a helical filament (Sung, 1997a, San Filippo et al., 2008).

**Pre-synapsis**

HR occurs through three main phases, termed presynapsis, synapsis and post-synapsis. The first phase, or pre-synaptic filament formation, begins when a nucleoprotein complex involving a recombinase (RAD51 or DMC1) is formed on the 3' end of the ssDNA tail (Figure 1.2). In the presence of ATP, the nucleoprotein complex
forms a right-handed helical filament to stretch the DNA to about twice the length of B-form dsDNA, which has 10.4 bases per helical turn (Sung and Robberson, 1995, Conway et al., 2004, Yu and Egelman, 2010). Linearization of the DNA molecule allows the search for homology between the chromosomes to begin. The presynaptic filament is then stabilized by accessory factors, such as RAD54 in humans or the SWI5-SFR1 complex in mice (Mazin et al., 2003, Tsai et al., 2012). Stabilization of the presynaptic filament stimulates the recombinase activity. Once a homologous region between the DNA is found, a displacement loop (D-loop) is formed in the template molecule, initiating synapsis (Bianco et al., 1998).
Figure 1.2 Presynaptic filament formation. After a DSB is created, the DNA is nucleolytically resected to produce a 3’ss tail which is coated with the ssDNA binding protein, RPA. Mediators and accessory factors, such as Brca2 in humans or Rad52 in yeast, assist the recombinase by removing RPA and promoting nucleoprotein complex formation on the ssDNA. The recombinase forms a right-handed helical filament on the ssDNA prior to displacement loop (D-loop) formation between the homologous chromosomes. RAD54 stabilizes the D-loop structure and removes RAD51 from the DNA before the DNA is replicated. 'S' designates sumoylated proteins. 'P' designates phosphorylated proteins. Adapted with permission from Krejci et al., 2012.
**Synapsis**

During synapsis, the D-loop is formed when the recombinase physically connects the invading ssDNA with the complementary DNA template (Bryant, 1984). The pairing of the template and donor duplex DNA molecule by the nucleoprotein complex creates the synaptic complex (Bianco et al., 1998). The nucleoprotein complex then performs strand exchange in an ATP-dependent manner between the two homologous chromosomes (Chi et al., 2006).

Mediators, such as Brca2 in humans and SWI5-SFR1 in *S. pombe*, function by stabilizing the nucleoprotein complex, loading the recombinase onto ssDNA or removing RPA-inhibition (Jensen et al., 2010, Haruta et al., 2006, Sung et al., 2003). Accessory proteins, such as RAD54 in humans, accelerate D-loop formation, stabilize RAD51 or DMC1 filament formation and stimulate ATP-hydrolysis (San Filippo et al., 2008, Mazin et al., 2003). In yeast, the mediator Rad52 assists RAD51 by alleviating RPA inhibition and stabilizing RAD51-filaments (Sung, 1997a, Seong et al., 2008).

**Post-synapsis**

After strand exchange, RAD54 dissociates RAD51 from the 3' OH through ATP hydrolysis, and polymerases utilize the homologous template to replicate the ssDNA (Solinger et al., 2002, Kiianitsa et al., 2006). Duplex products can then be resolved using three different mechanisms: double-strand break repair (DSBR), synthesis-dependent strand annealing (SDSA) or break-induced replication (BIR). Each mechanism follows a similar pathway during presynapsis and synapsis but resolves the joint duplex molecules differently (Figure 1.3A).
Figure 1.3 Resolution pathways of homologous recombination. (A). The 5' ends of the DSB are resected and bound by RPA. Mediators such as Rad52 in yeast remove RPA and assist the recombinase in presynaptic filament formation. The recombinase and accessory factors create a D-loop between the template and homologous chromosome during synapsis. The resolution of the D-loop is dependent on the HR pathway. (B.) In the double-strand break repair (DSBR) pathway, the D-loop is utilized to exchange DNA between the homologous chromosomes and results in a Holliday junction, which can be resolved in either a cross-over (CO) or non-crossover (NCO) event. (C.) During synthesis-dependent strand annealing
(SDSA), the D-loop is displaced and the homologous single-strand regions are annealed by accessory factors before DNA synthesis from polymerases. (D.) Break-induced repair (BIR) is utilized when only one end of the DSB is available to serve as a template for replication by DNA polymerases. (E.) Single-strand annealing (SSA) is the only HR pathway that does not require a recombinase and is considered to be mutagenic. Rad52 anneals the resected ssDNA regions prior to presynaptic filament formation. Adapted with permission from Krejci et al., 2012.

**Double-strand Break Repair (DSBR)**

The DSBR pathway can be available during mitosis but is predominately active during meiosis, where it is utilized to increase genetic diversity through crossing-over (Keeney and Neale, 2006). Numerous proteins are required to accurately and efficiently repair the DSB and ultimately exchange DNA through cross-over (CO) formation. However, CO formation results in new genetic material after exchange and is therefore only desired during meiosis, where it also establishes a physical link between the homologous chromosomes called the chiasma (Hotta et al., 1977). In mitotic cells, helicases such as Bloom (BLM) or RTEL1 in humans or Srs2 in yeast suppress CO formation, which could otherwise result in the loss of heterozygosity (LOH) (Wu and Hickson, 2003, Ira et al., 2003, Youds et al., 2010). Therefore, the DSBR pathway is primarily utilized only during meiosis, where it provides a mechanism for creating genetic diversity and proper segregation.

Post-synapsis in the DSBR pathway is tightly regulated (Figure 1.3B). After strand exchange mediated by the recombinase is completed, the second DNA molecule end is captured by Rad52 to stabilize the D-loop and extended through branch migration to create a double Holliday junction (dHj) (Shi et al., 2009). RAD54 expedites branch migration before removing RAD51 from the DNA molecule through ATP hydrolysis from both RAD51 and RAD54 (Bugreev et al., 2006, Li et al., 2007). The dHj is
resolved by helicases such as BLM or Werner (WRN) in humans or Sgs1 in yeast to either a CO or non-crossover (NCO) product prior to base filling by polymerases (Wu and Hickson, 2003, Cejka and Kowalczykowski, 2010). The extensive number of proteins in the DSBR pathway maintains strict regulation during meiosis to ensure crossing-over is completed accurately.

**Synthesis-dependent strand annealing (SDSA)**

The synthesis-dependent strand annealing (SDSA) pathway is the preferred DSB repair pathway in mitotic cells because the dissolution of the joint molecules results in NCO products; however, SDSA also functions in meiosis, providing an explanation for NCO events that occur in meiotic cells (Kadyk and Hartwell, 1992, Allers and Lichten, 2001, Merker et al., 2003). RTEL1 is unique to other helicases such as BLM or the yeast Srs2 due the ability of RTEL1 to disrupt a pre-formed D-loop (Barber et al., 2008). In an ATP-dependent manner, RTEL1 removes the template DNA molecule in the 5'-3' direction, allowing the ssDNA to re-anneal and reduce the length of the D-loop. The disruption of the D-loop prevents double Holliday junctions from occurring, resulting in NCO products (Cromie et al., 2006). RTEL1 also assists with branch migration of the D-loop structure until the 3' ssDNA region is released, allowing it to re-anneal with the homologous DNA strand. Only limited replication is needed by a polymerase to fill in the remaining gaps of the DSB (Figure 1.3C).
Break-Induced Repair (BIR)

Break-induced repair (BIR) often occurs when only one end of the DSB is available (Bosco and Haber, 1998). For example, when the DSB is significantly resected or at the end of a telomere, BIR is employed (Figure 1.3D). BIR uses the donor chromatid to replicate the DNA but requires extensive leading and lagging strand replication (Donnianni and Symington, 2013). LOH can occur in BIR due to repetitive sequences from nonhomologous chromosomes (Bosco and Haber, 1998).

Single-strand annealing (SSA)

The single-strand annealing (SSA) pathway of HR is utilized throughout the cell cycle to repair DSBs. Unlike the previous HR pathways, the SSA pathway does not require recombinase proteins or strand exchange to occur (Ivanov et al., 1996). Instead, the 5' end of the DSB is nucleolytically resected and bound by RPA. Rad52 then removes RPA and anneals the 3' ss overhangs (Figure 1.3E) (Mortensen et al., 1996). The SSA pathway is considered to be mutagenic because only a short region of the DNA must be complementary for Rad52 to anneal the strands (Stark et al., 2004).

Recombinases

Eukaryotes have two RecA-like recombinases, RAD51 and DMC1, and both recombinases have been extensively characterized in the HR pathway. While RAD51 has been identified in both mitotic and meiotic recombination, DMC1 is expressed only in meiosis (Shinohara et al., 1992, Bishop et al., 1992). However, genetic studies in yeast have shown that both RAD51 and DMC1 are required for meiotic recombination to occur.
(Cloud et al., 2012, Hong et al., 2013). Due to the function of RAD51 in mitosis and meiosis, it would be expected that deletion of *RAD51* would result in more significant abnormalities than deletion of *DMC1*. Indeed, *RAD51* deletion mutants do display severe phenotypes, such as the loss of spore viability (Shinohara et. al., 1992), while *DMC1* mutants show reduced spore viability (Bishop et. al., 1992). Similarly, deletion of *RAD51* in mice results in the extreme phenotype of embryonic lethality while *DMC1* null mutants in mice results in infertility (Lim and Hasty, 1996; and Pittman et al., 1998).

RAD51 has several recombination mediators, such as RAD52 and RAD55-57 in yeast and BRCA2 in humans, that function by assisting RAD51 during recombination (Sung, 1997a, b, San Filippo et al., 2006). Mediator functions include overcoming inhibition created by ssDNA binding proteins, loading recombinase proteins onto ssDNA, and assisting in strand exchange. RPA exhibits an inhibitory effect on strand exchange if allowed to remain bound to DNA, but RPA is required to prevent the ssDNA from forming secondary structures or reannealing (Sugiyama et al., 1997, Haruta et al., 2006, Sung, 1997a, Yuzhakov et al., 1999). The RPA inhibition prevents the formation of the nucleoprotein complex, thus stalling HR. Therefore, mediators function to remove RPA and load RAD51 onto the ssDNA.

Presynaptic filament formation and strand exchange activity by RAD51 and DMC1 require ATP binding but not hydrolysis (Chi et al., 2006; Sung and Stratton, 1996, Sharma et al., 2013). Both RAD51 and DMC1 contain two conserved ATP binding segments, Walker A and Walker B motifs, which are responsible for ATP-binding (Bishop et al., 1992, Shinohara et al., 1992). Sharma et al. (2013) constructed two ATP-
hydrolysis variants by mutating the conserved lysine of the Walker A motif in DMC1 (K132R and K132A) to demonstrate the importance of ATP binding. Interestingly, the K132R mutant was able to promote strand exchange in the presence of Ca$^{2+}$ ions. Calcium has previously been shown to stimulate and stabilize DMC1 filament formation and is proposed to induce a conformational change in the structure of DMC1 (Bugreev et al., 2005)

In the absence of DNA, RAD51 exists as a heptameric ring while DMC1 forms an octameric ring structure (Shin et al., 2003, Kinebuchi et al., 2004). Previous research suggested the active form of DMC1 was a circular ring instead of the helical filaments formed by RecA and RAD51 (Passy et al., 1999). In the presence of ATP, both RAD51 and DMC1 can form helical filaments on both ss and dsDNA, but only filaments on ssDNA are active (Benson et al., 1994, Bianco et al., 1998, Sehorn et al., 2004, Sung and Robberson, 1995).

Since the identification of DMC1 as a meiosis-specific recombinase protein (Bishop et al., 1992), several studies were aimed towards elucidating the mechanism of DMC1 in the meiotic recombination pathway. Most eukaryotes, with the exception of a few organisms such as D. melanogaster and C. elegans, contain the meiosis-specific RAD51 paralogue, DMC1 (Neale and Keeney, 2006). Although the lack of DMC1 in a few species indicates that DMC1 is not solely responsible for crossing over during meiosis, DMC1 is accountable for the majority of cross-over recombinants (Pittman et al., 1998, Cloud et al., 2012)
The first study to demonstrate the ability of DMC1 to perform robust strand exchange was conducted by Sehorn et al. (2004). Strand exchange activity of DMC1 increased significantly when both KCl concentration and pH were optimized. Importantly, the range of these conditions required strict adherence to physiological conditions, which would more accurately represent *in vivo* conditions. Additionally, the researchers were able to demonstrate that DMC1 activity is higher when ssDNA is presented before dsDNA. Demonstration of DMC1-mediated strand exchange activity supported the function of DMC1 as a recombinase.

Although compelling evidence supported the role of DMC1 as a recombinase, other functional differences between DMC1, RAD51 and RecA, such as filament structure, still existed. While the number of DMC1 filaments observed was similar to those produced by RAD51, DMC1 filaments still appeared to be shorter than RAD51 (Sung and Robberson, 1995). RAD51 produced filaments up to 50% longer than B-form DNA, which were significantly longer than those produced by DMC1. One possible explanation for why DMC1 filaments are shorter may be that all the required proteins, such as mediators and accessory proteins, are not yet present *in vitro*. For example, the *Schizosaccharomyces pombe* SWI5-SFR1 complex is required for elongation of filaments formed by the RAD51 homolog, spRHP51 (Kurokawa *et al*., 2008). Until recently, there were no known mediators for DMC1. However, the MEI5-SAE3 complex has now been shown to be a DMC1-specific mediator in *S. cerevisiae* (Ferrari *et al*., 2009).
Other studies that attempted to characterize filament formation of DMC1 are contradictory. Lee et al. (2005) reported that the average helical pitch of DMC1 filaments was approximately 50% longer than those formed by RAD51, or 24 nucleotides per turn of the DMC1 filament, in the presence of Ca\(^{2+}\) ions. In contrast, Sheridan et al. (2008) demonstrated DMC1 forms nucleoprotein filaments similar in length, helical pitch, filament diameter, and helical handedness to filaments produced by RAD51. This supported the view that DMC1 nucleoprotein filaments are indeed more similar to filaments produced by both RAD51 and RecA.

The importance of mediators and accessory proteins is clearly demonstrated by the interactions seen with RAD51 in mitotic recombination and both RAD51 and DMC1 in meiotic recombination. For example, RAD54 has been shown to disassemble RAD51 D-loops whereas DMC1-mediated D-loops are more resistant to RAD54 dissociation (Bugreev et al., 2011). Additionally, the scMEI5-SAE3 complex is required for Dmc1 strand exchange in the presence of RPA (Ferrari et al., 2009). These interactions are particularly important during meiosis, where DMC1 is responsible for generating recombinant DNA through cross-over formation.

**Single-strand DNA binding proteins**

**RPA**

Single-strand DNA binding (SSB) proteins are ubiquitous in cellular functions (Wold, 1997). Replication protein A (RPA) is the classical eukaryotic SSB protein and was first identified as a requirement for replication using the SV40 viral replication system (Wold and Kelly, 1998). RPA is heterotrimeric protein consisting of 70 kDa
(RPA1), 32 kDa (RPA2) and 14 kDa (RPA3) subunits. One of the defining structural characteristics of RPA (and other ssDNA binding proteins) is the presence of oligonucleotide/oligosaccharide binding (OB) folds in each subunit (Murzin, 1993). Structurally, OB folds consist of a five-stranded beta sheet that coils to form a closed beta barrel. RPA1 has four OB folds and displays the highest affinity for ssDNA while both RPA2 and RPA3 contain only one OB fold (Bochkarev et al., 1997, Bochkarev et al., 1999).

The high affinity of RPA for ssDNA and the ability to melt duplex DNA is important during DNA replication. RPA also assists in recruiting, stimulating processivity, and increasing the fidelity of polymerase α (Dornreiter et al., 1992, Braun et al., 1997, Maga et al., 2001). Polymerase α functions during the initiation phase of replication and synthesizes RNA-DNA primers of each Okazaki fragment (Waga and Stillman, 1994). Polymerase ε and δ function primarily during the elongation phase of replication, with Pol ε synthesizing on the leading strand and Pol δ on the lagging strand (Fukui et al., 2004). RPA also stimulates the activity of both polymerase ε and δ during elongation (Melendy and Stillman, 1991).

An interesting yet paradoxical observation concerning RPA in HR is that the protein inhibits recombinase nucleation on DNA yet also stimulates strand exchange by RAD51 and DMC1 (Sung and Robberson, 1995, Sehorn et al., 2004). The inhibitory effect of RPA is overcome through the use of mediators, such as Rad52 and Rad55-57 in yeast, by removing RPA from the ssDNA (Sung, 1997a,b). RPA also stimulates the
activity of other HR proteins, such as unwinding of dsDNA by the helicases BLM and WRN (Brosh et al., 1999, Brosh et al., 2000).

In 2008, two novel single-strand binding proteins were identified, human single-strand binding protein 1 and 2 (hSSB1 and hSSB2) (Richard et al., 2008). Interestingly, hSSB1 and hSSB2 are structurally more similar to the archeal SSB than to hRPA. However, like hRPA, both hSSB1 and hSSB2 are critical for the preservation of genomic integrity.

**hSSB1 and hSSB2**

Both hSSB1 and hSSB2 were identified based on homology to the crenarchaeon *Sulfolobus solfataricus* ssDNA-binding protein (SSoSSB) (Richard et al., 2008). While RPA is highly conserved in eukaryotes, the hSSBs are mainly found in vertebrates. hSSB1 (23kDa) and hSSB2 (22 kDa) share 59% identity, with the greatest diversity located in the C-terminal region. hSSB2 expression is increased when hSSB1 is decreased, and vice versa, which may indicate the hSSBs have overlapping yet distinct functions (Richard et al., 2008, Feldhahn et al., 2012).

Similarities between the hSSBs and RPA include a preference for ssDNA over dsDNA. Furthermore, hSSB1 and RPA protein levels are stabilized through phosphorylation by ATM kinase and phosphorylation occurs in response to radiation-induced DSBs (Wold, 1997, Richard et al., 2008). Like RPA, hSSB1 was also shown to stimulate RAD51-mediated D-loop activity *in vitro*, implying a role for hSSB1 in the HR pathway (Richard et al., 2008).
To identify the possible functions of hSSB1 in HR, Richard et al. (2008) utilized hSSB1 knockout cells. The mutants displayed a five-fold reduction in gene conversion compared to wild-type cells. Additionally, the researchers were able to show that RAD51 was unable to localize to DSBs in hSSB1-deleted cells. Further analysis of the hSSBs demonstrated that hSSB1 may result in cell cycle checkpoint defects, increased sensitivity to IR, and impaired HR (Richard et al., 2008). Interestingly, while hSSB1 formed foci at DSBs, hSSB1 functioned independent of cell-cycle phase, unlike RPA (Richard et al., 2008). RPA helps to regulate progression through the S-phase of the cell cycle and assists in halting the progression until the DSB is repaired (Wold, 1997). Given the differences between RPA and the hSSBs, it is likely that the two hSSBs act independently of RPA.

It has been reported that both hSSB1 and hSSB2 are part of the sensor of single strand (SOSS) DNA complex that binds to DSB ends and is required for ATM checkpoint signaling (Huang et al., 2009, Li et al., 2009). Both hSSB1 and hSSB2 form independent SOSS complexes with two proteins, Integrator Subunit 3 (INTS3) and human Single-strand Binding Interacting Protein 1 (hSSBIP1). Outside of the SOSS complex, hSSBIP1 has yet to be characterized. INTS3 was originally identified as one of 12 subunits in the Integrator complex, which assists in 3′ end formation and processing of small nuclear RNA (snRNA) (Baillat et al., 2005, Chen and Wagner, 2010). In the SOSS complex, INTS3 functions as a scaffolding protein by recruiting the hSSBs and hSSBIP1 in addition to regulating ATM activation, indicating that INTS3 plays a significant role in the SOSS complex (Li et al., 2009, Skaar et al., 2009).
In complex with INTS3 and hSSBIP1, hSSB1 was previously demonstrated to recruit and stimulate the MRN complex to DSBs via physical interaction with NBS1 (Richard et al., 2011a,b). However, recent genetic evidence has failed to support a role for hSSB1 or hSSB2 in ATM checkpoint activation or DSB processing (Feldhahn et al., 2012, Shi et al., 2013). Further studies are needed to determine if the SOSS complex is required for hSSB1 to influence checkpoint activation and stimulate MRN activity in vivo.

Two independent labs recently described the effects of hSSB1- and hSSB2-null mice, in which the embryos displayed perinatal lethality (Feldhahn et al. 2012, Shi et al., 2013). The embryos suffered from respiratory failure, skeletal defects and growth delays. Conditional deletion of hSSB1 in adult mice resulted in impaired male fertility and increased cancer susceptibility (Shi et al., 2013). Additionally, loss of hSSB2 but not hSSB1 in fibroblasts led to increased apoptosis and DSBs (Feldhahn et al., 2012). It is apparent that both hSSB1 and hSSB2 are critical for maintaining genomic integrity; however, additional studies are needed to further clarify the function of the hSSBs in HR.

**Mediators and Accessory Factors**

Mediators and accessory factors are required by RAD51 and DMC1 for HR to proceed efficiently. Mediators are defined by three distinct characteristics, including physical interaction with a recombinase, a high affinity for ssDNA and importantly, the ability to overcome RPA-inhibition (Sung et al., 2003). Relief of RPA-inhibition can occur by loading the recombinase onto free ssDNA, removing bound RPA from ssDNA or assisting the recombinase in helical filament formation on ssDNA (Shinohara et al.,
In humans, Brca2 is considered to function as a mediator to RAD51 while Rad52 is a mediator in yeast (Tarsounas et al., 2003, Jenson et al., 2010, Sung, 1997a, New et al., 1998). MEI5-SAE3 has been identified as a mediator to DMC1 in yeast but has not demonstrated in humans (Ferrari et al., 2009).

The role of accessory factors can range from stabilization of the presynaptic filament, stimulation of D-loop and/or strand exchange activity or removal of the recombinase from dsDNA after strand exchange (Mazin et al., 2003, Petukhova et al., 1998; Sigurdsson et al., 2002). RAD54 is a well-characterized and multi-functional example of an accessory factor to RAD51 in both humans and yeast (Mazin et al., 2003, Petukhova et al., 1998). For example, RAD54 can stimulate RAD51-mediated strand exchange through filament stabilization and utilizes ATP-hydrolysis to remove RAD51 from dsDNA when necessary (Mazin et al., 2003, Li et al., 2007).

**Brca2**

In mammalian cells, the breast cancer susceptibility gene 2 (BRCA2) plays a major role in RAD51 localization to DSBs (Tarsounas et al., 2003). BRCA2 is a tumor suppressor implicated in breast and ovarian cancer (Yu et al., 2000, Thorslund and West, 2007). Recombinant full-length BRCA2 was only recently purified and shown to promote RAD51-mediated recombination by targeting RAD51 to RPA-coated ssDNA (Jenson et al., 2010, Liu et al., 2010).

Structurally, full-length BRCA2 is a large protein (3,418 aa) and has three OB folds that function in both DNA binding and numerous protein interactions. Protein interactions help regulate BRCA2 function. For example, DSS1 (deleted in spilt
hand/spilt foot) binds to one of the OB folds and stabilizes BRCA2 expression in response to DSB damage (Yang et al., 2002, Li et al., 2006). Additionally, the tumor suppressor PALB2 interacts with BRCA2 and assists with recruiting RAD51 to DSBs (Dray et al., 2010).

**RAD52**

In yeast, RAD52 is required for RAD51 recruitment to DSBs and functions as a classical mediator to RAD51, in that RAD52 can remove RPA ssDNA and assist RAD51 filament formation (Gasior et al., 1998, Sung, 1997a). RAD52 mediator activity is dependent upon the interaction with RAD51 and RPA (Krejci et al., 2002, Plate et al., 2008). RAD52 promotes second-end capture of D-loop molecules and anneal RPA-coated ssDNA in addition to stimulating polymerase ETA activity during post-synapsis (Mortensen et al., 1996, Nimonkar et al., 2009, Mcllwraith and West, 2008). However, RAD52 appears to be more critical in yeast than humans, as RAD52 mediator activity in humans as not yet been shown.

**The MEI5-SAE3 complex in yeast**

Early genetic studies implicated both MEI5 and SAE3 in HR through a screen designed in yeast to identify meiotic recombination proteins that displayed similar phenotypes to DMC1-null mutants (Hayase et al., 2004, Tsubouchi and Roeder, 2004). In 2004, Hayase et al. produced both MEI5 and SAE3 null mutants in a rapidly sporulating background, the SK-1 line. The hypothesis that MEI5-SAE3 was required for DMC1 to assemble on DNA was supported by the finding that both MEI5 and SAE3 null
mutants resulted in phenotypes similar to DMC1-null mutants, including arrest during prophase, accumulation of DSBs at recombination hotspots, reduced sporulation and spore viability. The MEI5 mutant also displayed fewer cross-over recombinants than wild type. The mutant phenotypes of both the MEI5 and SAE3 mutants were rescued by overexpression of RAD51 (Hayase et al., 2004, Tsubouchi and Roeder, 2004). Implications from the study involving MEI5-SAE3 suggested that the complex functioned in HR as a loading factor specific to DMC1 (Hayase et al., 2004).

In addition to DMC1, the MEI5-SAE3 complex also appears to assist RAD51 during HR, albeit using a different mechanism. For example, while DMC1 was incapable of binding the DNA in the absence of MEI5 and SAE3, RAD51 would readily bind the DNA (Hayase et al., 2004). Additionally, RAD51 was unable to dissociate from the DNA in the absence of MEI5 and SAE3, suggesting MEI5-SAE3 may play a role in RAD51 removal after strand exchange (Hayase et al., 2004). However, it is likely that proteins other than MEI5-SAE3 remove RAD51 from DNA. For example, Rdh54, a RAD54 homolog in S. cerevisiae, has been shown to both interact and remove RAD51 from dsDNA, perhaps in an effort to prevent non-specific binding by the recombinase in the early phase of HR (Chi et al., 2009). Regardless, it appears the MEI5-SAE3 complex may assist RAD51 and DMC1 through different mechanisms.

In meiotic recombination, DSBs are induced by the topoisomerase Spo11. Although Hayase et al. (2004) demonstrated MEI5 and SAE3 localization to DSBs, Tsubouchi and Roeder (2004) was able to show that the proteins localize in a Spo11-dependent manner, indicating that MEI5 and SAE3 function during meiotic HR.
Furthermore, DMC1, MEI5 and SAE3 were shown to be dependent upon each other for localization to recombination hotspots. Taken together, MEI5 and SAE3 appear to function in the meiotic recombination pathway in yeast.

In 2006, Haruta et al. reported that in vitro strand exchange activity by S. pombe DMC1 was slightly increased when spSWI5-SFR1, a homologue of MEI5-SAE3, was incorporated. However, the increase in activity was not significant enough to support SWI5-SFR1 as a mediator in S. pombe. Recently, Murayama et al. (2013) provided compelling support for spSWI5-SFR1 mediator activity to DMC1. spSWI5-SFR1 can both load spDMC1 onto RPA coated ssDNA and stimulate strand exchange activity by the recombinase; therefore, spSWI5-SFR1 functions as a mediator to spDmc1.

In S. cerevisiae, Ferrari et al. (2009) demonstrated that the MEI5-SAE3 complex functions as a mediator to scDMC1 during meiotic recombination. The DNA binding characteristics indicated that scMEI5-SAE3 has the capability to bind both long ssDNA and dsDNA, with a substantial preference for ssDNA. After further analysis of the DNA binding activity of scMEI5-SAE3, it was determined that scMEI5-SAE3 preferentially binds forked substrates over ssDNA (Say et al., 2011). The N-terminal region of scMEI5 has been shown to interact with scDMC1 using two-hybrid analysis and scRAD51 through physical interaction (Hayase et al., 2004, Say et al., 2011). The scMEI5-SAE3 also has the ability to overcome the inhibitory effect of RPA and load DMC1 onto ssDNA (Ferrari et al., 2009). However, scMEI5-SAE3 acts as a mediator to DMC1 specifically and not RAD51 (Say et al., 2011). It may be that, like spSWI5-SFR1, scMEI5-SAE3 is not the optimal protein to remove RPA for RAD51. Additional studies
are needed to identify mediators or accessory factors that may influence MEI5-SAE3 activity on RAD51.

Recently, it was reported that *M. musculus* SWI5-SFR1 complex stimulates ATP hydrolysis of RAD51 *in vitro* during presynaptic filament formation and stabilizes the RAD51 filament by enhancing ADP release (Su et al., 2013). This is in contrast to the yeast orthologs, which seem to function predominately with Dmc1. However, the mSWI5-SFR1 complex appears to function using a different mechanism than the yeast orthologs, as mSWI5-SFR1 does not bind DNA (Tsai et al., 2012).

Mediator proteins interact with both a recombinase and a SSB protein, such as RPA. One such example includes the Rad52 interaction with both RAD51 and RPA (Shinohara et al., 1992). Ferrari et al. (2009) used co-immunoprecipitation, along with ssDNA-bound magnetic beads, to demonstrate that MEI5-SAE3 interacts with both DMC1 and RPA. This interaction appears to be similar to that of Rad52, in that MEI5-SAE3 has the ability to bind both a recombinase and a SSB protein. However, Rad52 can also anneal ssDNA whereas MEI5-SAE3 is unable to anneal complementary DNA (Say et al., 2011).

**Human MEI5-SWI5 complex**

Recently, the human orthologs of the yeast MEI5-SAE3, the hMEI5-SWI5 complex, were identified (Yuan and Chen, 2011). The MEI5-SWI5 complex is conserved across eukaryotes (Figure 1.4); however, several differences are evident between the human MEI5-SWI5 and *S. cerevisiae* MEI5-SAE3, *S. pombe* SWI-SFR1 and *M. musculus* SWI5-SFR1 (Table 1.1). MEI5-SAE3 expression in *S. cerevisiae* is the
only homologue that is meiosis-specific while MEI5-SWI5 is expressed during both mitosis and meiosis in *S. pombe* (Haruta *et al.*, 2006, Yuan and Chen, 2011). Human MEI5 and SWI5 both physically interact with RAD51 while only MEI5 in yeast and SFR1 in mice interact with RAD51 (Yuan and Chen, 2011, Say *et al.*, 2011, Akamatsu and Jasin, 2010). Additionally, RAD51 localization to the site of DSBs is reduced in the absence of human MEI5-SWI5 in contrast to the loss of MEI5-SAE3 in yeast, where RAD51 foci localizes and remains at the break (Yuan and Chen, 2011, Hayase *et al.*, 2004).

![Figure 1.4](image.png)

**Figure 1.4 MEI5-SWI5 are evolutionarily conserved among eukaryotes.** MEI5 domains are shown in blue, and SWI5 domains are green. Conserved coiled-coil (cc) domains are shown in orange. Adapted with permission from Yuan and Chen, 2011).

<table>
<thead>
<tr>
<th>Mei5 homologues</th>
<th>Swi5 homologues</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H. sapiens</strong></td>
<td></td>
</tr>
<tr>
<td>Mei5 domain</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td></td>
</tr>
<tr>
<td>232</td>
<td></td>
</tr>
<tr>
<td><strong>M. musculus</strong></td>
<td></td>
</tr>
<tr>
<td>Mei5 domain</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td></td>
</tr>
<tr>
<td>319</td>
<td></td>
</tr>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td></td>
</tr>
<tr>
<td>Mei5 domain</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td></td>
</tr>
<tr>
<td>222</td>
<td></td>
</tr>
<tr>
<td><strong>S. pombe</strong></td>
<td></td>
</tr>
<tr>
<td>Mei5 domain</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td></td>
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<tr>
<td>299</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.1. Similarities and differences between the eukaryotic MEI5-SWI5 homologues.** Expression pattern, RAD51 interaction and activity, and DMC1 interaction and activity are compared.

<table>
<thead>
<tr>
<th>Expression</th>
<th><em>H. sapiens</em> Mei5-Swi5</th>
<th><em>M. musculus</em> Swi5-Sfr1</th>
<th><em>S. cerevisiae</em> Mei5-Sae3</th>
<th><em>S. pombe</em> Swi5-Sfr1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rad51 Interaction</td>
<td>Mei5-Swi5</td>
<td>Sfr1</td>
<td>Mei5</td>
<td>Swi5-Sfr1</td>
</tr>
<tr>
<td>Rad51 Activity</td>
<td>Unknown</td>
<td>Accessory</td>
<td>Accessory</td>
<td>Mediator</td>
</tr>
<tr>
<td>Dmc1 Interaction</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Mei5</td>
<td>Swi5-Sfr1</td>
</tr>
<tr>
<td>Dmc1 Activity</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Mediator</td>
<td>Mediator</td>
</tr>
</tbody>
</table>
**RAD54**

RAD54 has multiple functions in the HR pathway, beginning with stabilization of RAD51 presynaptic filament formation (Mazin *et al*., 2003). Furthermore, RAD54 stimulates D-loop formation and strand exchange of RAD51 (Petukhova *et al*., 1998; Sigurdsson *et al*., 2002). RAD54 is also a DNA translocase with dsDNA-dependent ATPase activity (Busygina *et al*., 2008, Petukhova *et al*., 1998). The translocase activity is thought to facilitate the search for homology before synapsis (Petukhova *et al*., 1998, Van Komen *et al*., 2000). However, interaction with RAD51 during the post-synaptic phase stimulates the ATPase and DNA branch migration activity of RAD54, dissembling RAD51 from the dsDNA (Li *et al*., 2007, Rossi and Mazin, 2008, Zhang *et al*., 2007). To date, no functional interaction has been observed between RAD54 and DMC1.

**DNA Synthesis in Homologous Recombination**

In humans, at least 15 polymerases have been identified and are grouped based on activity in 4 different families. While most polymerases display high fidelity during replication, translesion synthesis (TLS) polymerases have low fidelity and can replicative through large adducts on DNA such as thymine-dimers (Masutani *et al*., 1999). DNA polymerases, including δ, η and κ, function during post-synapsis in several HR pathways, including DSBR, SDSA and BIR (Figure 1.5) (Sebesta *et al*., 2013).
**Figure 1.5 Polymerases in HR.** In response to stalled replication forks or DSBs, polymerases Delta, Eta or Kappa (shown in yellow) are recruited to replicate DNA. Polymerase Delta is more processive and can extend further across the DNA. Both polymerase Eta and Kappa are error-prone and are only utilized to extend short DNA sequences. PCNA (shown in blue) stimulates polymerase activity. Final products can be resolved through DSBR, SDSA or BIR mechanisms. Adapted with permission from Sebesta et al., 2013.
**hPolymerase η**

Human polymerase η is a Y-family TLS polymerase that can accurately synthesize through bulky DNA lesions or through a collapsed replication fork (Plosky and Woodgate, 2004). Loss of hPol η has been implicated as the cause of Xeroderma Pigmentosum variant (XP-V) syndrome, which is characterized by extreme sensitivity to sunlight (Johnson et al., 1999). Thymine-dimers caused by UV exposure are not efficiently repaired in the absence of Pol η, leading to a high risk of skin cancer, indicating the importance of Pol η in DNA repair (Masutani et al., 1999).

Although hPol η is considered to be a relatively error-free TLS polymerase, the activity of hPol η is tightly regulated (Prakash et al., 2005). Regulation of TLS polymerases is achieved through post-translational modifications of the proliferating cell nuclear antigen (PCNA) (Lee and Myung, 2008). For example, after DNA damage, PCNA is monoubiquinated to increase the affinity between the clamp and hPol η (Bienko et al., 2005). PCNA, replication factor C (RFC) and RPA all work concertedly to stimulate the polymerase activity of hPol η on circular ssDNA (Haracska et al., 2001).

In addition to TLS activity, evidence supports a role for hPol η in the later stages of HR. Kawamoto et al. (2005) first implicated Pol η in DSB repair after deletion of the gene in chickens led to reduced DSB-induced gene conversion. Overexpression of hPol η resulted in a 5-fold increase in HR (Sebesta et al., 2013). Additionally, hPol η has been shown to preferentially bind and synthesize DNA from D-loop structures (Mcllwraith et al., 2005, Mcllwraith and West, 2008). Unlike hPol δ, PCNA has no effect on the ability...
of hPol η to synthesize from D-loop structures, suggesting that PCNA may regulate polymerase localization to HR substrates (Sebesta et al., 2013).

Summary

The homologous recombination pathway (HR) is responsible for ensuring genomic integrity, and accordingly, the HR pathway is complex. Many of the intricate details regarding the recombinase proteins, their mediators and accessory factors have yet to be identified. RPA and hSSB1/2 have roles throughout the HR pathway and diverse functionality. Although there are many studies regarding the hSSB proteins, more details are needed to refine the role of the hSSBs in HR. Here, I demonstrate a novel activity of the hSSBs and provide a functional mechanism for this activity in HR and DSB repair. In addition, I have purified INTS3 and hSSBIP1 and demonstrated physical interaction with the hSSB proteins. The work presented on hSSB1 and hSSB2 indicates both proteins may function in the repair of stalled replication forks in addition to HR.

Although the yeast orthologs of hMEI5 and hSWI5 are mediators to DMC1, hMEI5-SWI5 function with hDMC1 has not yet been determined. Based on the activity of the yeast and mouse homologs of MEI5-SWI5, it is likely that hMEI5-SWI5 contributes to both RAD51- and DMC1-mediated activity. In Chapter 4, I have characterized the hMEI5 and hSWI5 proteins and demonstrated that hMEI5 but not hSWI5 retains the ability to function as a mediator to hDMC1.

References


CHAPTER 2
HSSB1 AND HSSB2 STIMULATES POLYMERASE η EXTENSION FROM D-LOOP STRUCTURES

Abstract

Single-strand DNA binding proteins are required in numerous cellular functions including DNA replication, repair and recombination. The role of replication protein A (RPA) has been extensively characterized in all three mechanisms, whereas hSSB1 and hSSB2 were first implicated in DNA repair and maintenance of genomic integrity. Recent in vivo data provided evidence that hSSB1 and hSSB2 has a role in genomic maintenance and replication (Feldhahn et al., 2012). In this study, we have demonstrated a unique activity of hSSB1 and hSSB2 that could be utilized during DNA repair and replication. Specifically, both hSSB1 and hSSB2 can anneal complementary ssDNA and, like other SSBs, melt duplex DNA. Additionally, a physical and functional interaction of the hSSBs with Polymerase η was identified.

1. Introduction

In an effort to maintain genomic integrity, DNA predominantly exists as double-stranded (ds) molecules. However, single-strand (ss) DNA is required during replication or arises from DNA damage such as DNA ds breaks (DSBs). Single-strand binding (SSB) proteins are ubiquitous in cellular functions, including DNA replication, repair and recombination and are conserved in all known organisms (Wold, 1997, Szczepanska et al., 2007, Richard et al., 2009). The common structural feature of SSBs is an
oligonucleotide-binding (OB) fold that has a high affinity for ssDNA. SSBs rapidly localize to and bind ssDNA to provide protection from degradation or modifications during replication, DNA repair and recombination.

RPA is the classical eukaryotic SSB and is critical for cell viability (Wold, 1997, Wang et al., 2005). Loss of RPA in mice is embryonic lethal while even heterozygous mice display an increased susceptibility to tumor formation and chromosomal breaks (Wang et al., 2005). Two additional human SSB proteins, hSSB1 and hSSB2, have also been characterized in DNA repair and recombination (Richard et al., 2008, Huang et al., 2009, Li et al., 2009, Skaar et al., 2009). Initial analysis of the hSSBs suggested a role in homologous recombination (HR) through ATM (ataxia telangiectasia-mutated) activation, RAD51 localization to DNA DSBs and DSB processing through stimulation of the MRN complex (Richard et al., 2008, Richard et al., 2011a,b). Recently, the mouse homologues of the hSSBs were implicated in the protection of newly replicated telomeres through a physical interaction with the ssDNA binding protein protection of telomere 1a (Pot1a) (Gu et al., 2013). Additionally, the loss of mSSB1 and mSSB2 resulted in a significant increase in chromatid fusions involving both leading- and lagging-strand telomere ends (Gu et al., 2013).

Interestingly, SSB1 and SSB2-null mice are perinatal lethal, resulting from respiratory failure and skeletal defects. Conditional deletion of either SSB in adult mice lead to increased sensitivity to ionizing radiation, cancer susceptibility and impaired male fertility (Feldhahn et al., 2012, Shi et al., 2013, Gu et al., 2013). Furthermore, loss of hSSB2 in fibroblasts leads to increased apoptosis, DSBs and defects in proliferating cells,
suggesting that hSSB2 is essential for replication (Feldhahn et al., 2012). However, protein expression of hSSB1 and hSSB2 are co-regulated, as the loss of one leads to an increase in the other, suggesting an overlap of function between the two SSBs (Richard et al., 2008, Feldhahn et al., 2012, Gu et al., 2013).

One of the interesting aspects of the hSSBs is that both were identified based on homology to the archeal SSB but not RPA in humans. Importantly, the archaean Sulfolobus solfataricus SSB functions in a similar manner as RPA but has been shown to possess efficient dsDNA strand melting activity (Cubeddu and White, 2005). RPA also retains the ability to melt duplex DNA but only stimulates ssDNA annealing if the DNA has secondary structure, preferably 3' ssDNA overhangs (Bartos et al., 2008, Delagoutte et al., 2011). RPA accelerates the annealing by preventing the ssDNA strands from folding over, forming hairpin structures (Bartos et al., 2008, Chen et al., 2013). hSSB1 also has been shown to possess duplex DNA melting activity but without specificity to substrate structure (Delagoutte et al., 2011).

The human polymerase η is a DNA translesion-synthesis (TLS) polymerase that functions in HR. Several studies have provided support for the role of hPol η in D-loop extension and the repair of stalled replication forks (McIlwraith and West, 2008, Sebesta et al., 2013, Sneeden et al., 2013). Recently, the hSSBs have been implicated in the repair of stalled replication forks (Bolderson et al., 2014). We therefore hypothesized that the hSSBs may assist hPol η during HR and the replication of stalled replication forks.
In this report, we demonstrate that the hSSBs have ssDNA annealing activity and duplex melting activity through homologous DNA pairing. Surprisingly, we also identified a functional interaction with hPol η. Specifically, the hSSBs stimulate hPol η extension on synthetic oligonucleotide D-loop structures in addition to D-loop extension using plasmid-length substrates. These results support a function for hSSB1 and hSSB2 in HR and during the repair of stalled replications forks.

2. Materials and Methods

2.1 Phylogenetic Inference of Single Strand DNA Binding Proteins

2.1a Sequence Retrieval and Alignment

We obtained the accession numbers for the SSB proteins from representative organisms of the three domains of life (Escherichia coli SSB: P0AGE0, Sulfolobus solfataricus SSB: AAK42515, Homo sapiens SSB1: Q9BQ15 and Homo sapiens SSB2: Q96AH0). For each sequence, one iteration of the PSI-BLAST search was completed using the default parameters. The maximum number of retrieved sequences was set at 500. Each raw dataset was clustered using BLASTClust (Altschul et al., 1997). The maximum identity threshold was 85% and sequence coverage of 100%. The accession number selected for each cluster was based on the largest sequence within that cluster, and a manual analysis of the sequences further curated the dataset. Each of the datasets was merged into one, comprising 251 sequences from Archaeal, Eubacterial and Eukaryotal organisms. Additionally, human replication factor 3 (P35244) was incorporated prior to the analysis as an outgroup sequence. The sequences were aligned
at default (ClustalW format with aligned output) using the MUSCLE algorithm incorporated in MEGA5 (Edgar, 2004, Tamura et al., 2011).

2.1b Maximum Likelihood Phylogenetic Tree

The alignment file was used to calculate the amino acid substitution model for the phylogenetic reconstruction. The best model was WAG+G, determined by using the incorporated tool in MEGA5 (Tamura et al., 2011). The model was incorporated into the Maximum Likelihood reconstruction, with the treatment for gap penalties and missing data set to partial deletion and a cutoff of 90%. The selected test of phylogeny was bootstrapped with 200 replications. We also tested the accuracy of the tree by selecting the second and third best substitution models. No discrepancies between the trees were identified. The tree file was then exported to MESQUITE for visualization and editing (Maddison and Maddison, 2011).

A further refined tree was created using the 5 sequences identified in the larger phylogeny to be within the node that contained the reference entries for hSSB1 and hSSB2 as well as Sulfolobus solfataricus. These sequences, as well as the outgroup, were extracted from the curated dataset and aligned. The best amino acid substitution model was identified as JTT (a modified version of Dayhoff PAM matrices). The phylogeny was reconstructed using MEGA5 with the same parameters applied to the larger dataset.
2.2 Protein Purification

2.2a hSSB1 and hSSB2 purification protocol

The hSSB1 expression plasmid was transformed into *E. coli* Rosetta (DE3) strain cells, grown at 37°C to an $A_{600}$ of ~ 1.0 prior to the addition of 0.4 mM IPTG and incubated for ~ 20 h at 16°C. The cells were harvested by centrifugation using a Beckman JLA 16.250 rotor at 4500 g at 4°C. All subsequent steps were carried out at 4°C. 30 g cell paste was resuspended in Buffer A (50 mM Tris pH 7.5, 1 mM EDTA, 10% sucrose, 0.01% Igepal, 1 mM β-mercaptoethanol, 0.1 mg/mL lysozyme, 1 mM benzamidine, 1 mM PMSF, and protease inhibitors: aprotinin, chymostatin, leupeptin, and pepstatin A each at a final concentration of 5 µg/mL) containing 150 mM KCl and subjected to sonication at a constant output at of 4 (3 times at 30 second cycles). The extract was clarified by ultracentrifugation in a Beckman Ti.45 rotor at 100,000 g for 90 min. The supernatant was diluted 1:3 in Buffer B (20 mM KH$_2$PO$_4$ pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, and 1 mM β-mercaptoethanol) and loaded onto a Q-sepharose column (GE Healthcare). The protein was fractionated in Buffer B with a gradient from 100 – 700 mM KCl. Fractions containing hSSB1 were determined by Coomassie staining and diluted 1:2 in Buffer B containing 300 mM KCl and 40 mM imidazole before incubated with 1 mL Ni-NTA Sepharose 6 Fast Flow beads (GE Healthcare) overnight. The flow was collected prior to washing the beads with 10 mL Buffer B containing 1 M KCl and 40 mM imidazole followed by a wash with 10 mL of Buffer B containing 300 mM KCl and 40 mM imidazole. The protein was eluted with Buffer J containing 300 mM KCl and 500 mM imidazole. Eluted fractions were then
pooled and diluted 1:4 with Buffer B before loaded onto a 1 mL Macro Hydroxyapatite column (Bio-Rad). The protein was fractionated with Buffer B containing 100 mM KCl and 0 – 300 mM KH$_2$PO$_4$ gradient with 400 mM KCl. Peak fractions (~150 mM KH$_2$PO$_4$) were pooled, diluted 1:4 with Buffer B and loaded onto a 1 mL Source S column (GE Healthcare). The protein was fractionated with a 20 mL gradient of Buffer B containing 100 mM – 500 mM KCl and peak fractions (~200 mM KCl) were pooled and concentrated to 10 mg/mL in a Centricon-10 concentrator. Aliquots of the purified protein were stored at -80°C. hSSB2 was expressed and purified following the same protocol as hSSB1.

2.2b hPolymerase η Expression and Purification

The hPol η bacterial expression plasmid, a kind gift from Zucai Sou (Ohio State University), in the pET-21b plasmid harboring a C-terminal (HIS)$_6$ tag. The pET21-hPolη vector was transformed into E. coli Rosetta (DE3) cells, grown at 37°C to an $A_{600}$ of ~ 1.0 before the addition of 0.4 mM IPTG. After an incubation for ~ 20 h at 16°C, the cells were harvested by centrifugation using a Beckman JLA 16.250 rotor at 4500 g at 4°C. Cell paste (60 g) was resuspended in Buffer A containing 250 mM KCl and subjected to sonication 3 times for 30 seconds at a constant output at of 6. The extract was clarified by ultracentrifugation using a Beckman Ti.45 rotor at 100,000 g for 90 min. The supernatant was incubated with 0.5 mL Ni-NTA Sepharose 6 Fast Flow beads (GE Healthcare) overnight in Buffer B containing 300 mM KCl and 40 mM imidazole. The flow-through was collected prior to washing the beads with 5 mL Buffer B containing 1 M KCl and 40 mM imidazole followed by a wash with 5 mL of Buffer B containing 300
mM KCl and 40 mM imidazole. The protein was eluted with Buffer B containing 300 mM KCl and 500 mM imidazole. Eluted fractions were then pooled, diluted 1:4 with Buffer B and loaded onto a 1 mL Macro Hydroxyapatite column (Bio-Rad). The protein was fractionated with Buffer B containing 100 mM KCl and 0 – 300 mM KH$_2$PO$_4$ with 500 mM KCl. Peak fractions (~180 mM KH$_2$PO$_4$) were pooled, diluted 1:3 in Buffer B, and passed across a 1 mL Source S column (GE Healthcare) and washed with 5 column volumes of 100 mM KCl. The column was developed with a 30 mL gradient of Buffer B containing 100-600 mM KCl. Peak fractions were then pooled and concentrated to 200 µL in a Centricon-30 concentrator (Millipore). The protein was applied to a 20 mL Sephacryl S-200 column (0.9 x 30 cm) equilibrated in Buffer B containing 100 mM KCl. The proteins were fractionated at 0.25 mL/min in Buffer B with 100 mM KCl. Peak fractions were pooled and concentrated to 5 mg/mL in a Centricon-30 concentrator. Aliquots of the purified protein were stored at -80°C.

2.3 DNA Substrates

Oligonucleotide (OL) 83 (Table 2.1) was labeled with [γ-³²P]-ATP using T4 polynucleotide kinase (New England Biolabs). Unincorporated [γ-³²P] ATP was removed using Micro Bio-Spin 30 Columns (Bio-Rad). All other oligonucleotides used in this study were radiolabeled following the same procedure (Table 2.1).

2.4 Single-Strand Annealing Assay

Unlabeled OL83-c (0.83 µM nucleotides each) was incubated with hSSB1 (0.27 µM), hSSB2 (0.18 µM), hRPA (0.18 µM), scRad52 (0.34 µM) or scRPA (0.18 µM) at
37°C in the presence in Buffer C (50 mM Tris–HCl pH 7.4, 1 mM DTT) for 5 min. The annealing reaction was initiated by the addition of $^{32}$P-OL83 (0.83 μM nucleotides each) and incubated at 37°C. At the indicated times, 2 μL aliquots were removed and quenched by the addition of 10-fold excess of unlabeled OL83-c prior to deproteinization by treatment with Proteinase K (0.5 mg/mL) and SDS (0.8% final) for 10 min at 37°C. The samples were subjected to 12% non-denaturing Tris-acetate-EDTA (TAE) polyacrylamide gel electrophoresis. The gels were dried, analyzed with a Typhoon phosphorimager and quantified with ImageQuant (GE Healthcare) software. All gels used in this study were analyzed using the same technique unless otherwise stated.

**Table 2.1** Oligonucleotide sequences used in ssDNA annealing, DNA pairing, and D-loop.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLH3</td>
<td>TTGATAAGAGGTCATTTGAATTCATGGCTTAGCTTAATTGCTG AATCTGGTGCTGGGATCCAACATGTTTTAAATATG</td>
</tr>
<tr>
<td>OLH3-c</td>
<td>CATATTTAAAAACATGTGATTCAGCACCAGATTCAGCAATTA AGCTCTAAGCATGAATTCAAATGACCTCTATCAAA</td>
</tr>
<tr>
<td>OL80</td>
<td>AAAAAACCACCGCTACCAAGCGGTGGTTGGGCTGCGATCAAGAG CTACCAACTCTTTTTCCGAAGGTAACCTGCTTCAGC</td>
</tr>
<tr>
<td>OL83</td>
<td>AAATGAAACATAAGTAAAGTATAAGGATAATACAAAAAATAA GTAAATGAAATAAACATAGAAATAAAGTAAAGGATATAAA</td>
</tr>
<tr>
<td>OL83-c</td>
<td>TTTATATCTCTTTACTTTTATATTTCTATGTTCTAATTCTTTACTTTGATTT</td>
</tr>
<tr>
<td>OL90</td>
<td>AAATCAATCTAAAAGTATAATATGAGTAAAACCTGGTCTGACAGTTACCAA TGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATT</td>
</tr>
<tr>
<td>OL90-c</td>
<td>AATAGACAGATCTCGGAGTAGGTGGCTCAGCTCATGAAGCATTG GTAACCTGCTAGACAAGGTTTACTCATATATAGTTGATTT</td>
</tr>
</tbody>
</table>
2.5 Oligonucleotide-based homologous DNA pairing assay

To construct the duplex DNA, the 5'-end of OL83 was labeled with [γ-32P]-ATP using T4 polynucleotide kinase (New England Biolabs). Unless otherwise stated, all oligonucleotides were annealed and purified as follows. Annealing of 32P-OL83 and unlabeled OL83-c was accomplished by heating to 100°C for 5 min in Buffer D (100 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM EDTA pH 8.0) before slow cooling and gel purification.

To detect homologous DNA pairing, unlabeled OL83 (10 µM nucleotides) was incubated with 0.45 µM hSSB1 or 0.28 µM hSSB2 in Buffer C. The duplex DNA composed of 32P-labeled OL83 annealed to OL83-c (5µM base pairs) was added with 1 µL of 50 mM spermidine to the reaction mixture and further incubated at 37°C for the indicated times (final reaction volume 12.5 µL). An aliquot (2 µL) was withdrawn at different time points, deproteinized by treatment with Proteinase K (0.5 mg/mL) and SDS (0.8% final) at 37°C for 10 min. The reaction products were subjected to 12% native polyacrylamide gel electrophoresis in TAE buffer.

2.6 D-loop Assay

The 32P-labeled D-loop substrate (OL90, 2.5 µM nucleotides) was incubated with 1.5 µM hSSB1 or 0.8 µM hSSB2 in Buffer C (final reaction volume 12.5 µL) for 10 min. The reaction was initiated by addition of pBluescript SK replicative form I (35 µM base pairs). At the indicated times, a 2 µL aliquot was withdrawn and deproteinized by treatment with Proteinase K (0.5 mg/mL) and SDS (0.8% final) at 37°C for 10 min. The products were separated using electrophoresis on a 1.0% agarose gel in TAE buffer.
2.7 Second-end capture and D-loop stabilization

To detect second-end capture, the D-loop assay (as described in section 2.6) was performed in full. After the 10 min incubation with pBluescript (35 μM base pairs), complementary unlabeled OL90-c was added to the reaction and incubated for an additional 10 min before 1 unit of EcoR1 (as indicated) and 1 μL of Buffer E (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM MgCl₂) were added. The reaction was further incubated at 37°C for 45 min. Reaction products were deproteinized at 37°C for 10 min before being subjected to 1.0% agarose gel electrophoresis in TAE buffer.

2.8 Pull-down assays

hSSB1 (3 mg) or hSSB2 (3 mg) or BSA (6 mg) were immobilized on 1 mL of Affi-Gel 15 (Bio-Rad) per the manufacturer's instructions and stored in Buffer F (50 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 30% glycerol) at -20°C. The indicated Affi-Gel matrices were equilibrated with Buffer G (20 mM KH₂PO₄ pH 7.5, 1 mM DTT, 1% Triton, 10% glycerol) containing 100 mM KCl. hPol η (3 μg) was added to the indicated Affi-gel matrix in 30 μL of Buffer G with a final concentration of 100 mM KCl and agitated for 30 min at 4°C. The supernatant was removed followed by three washes of the beads with Buffer G containing 100 mM KCl before 30 μL of 2x SDS loading dye was added each fraction. The supernatant, wash and bead samples were incubated at 95°C prior to loading 8 μL of each onto a 12% SDS-PAGE followed by Coomassie Blue staining.
2.9 D-loop extension

The D-loop assay (described in section 2.6) was also utilized to examine DNA polymerase activity. Briefly, $^{32}$P-OL90 (2.5 µM nucleotides) was incubated with 1.5 µM hSSB1 or 0.8 µM hSSB2 in Buffer C for 10 min prior to the addition of pBluescript SK replicative form I (35 µM base pairs). After a 10 min incubation at 37°C, a 2 µL aliquot was taken as the starting time point before hPol η (0.25 µM) and 1.5 µL Buffer H (0.125 mM dATP, dCTP, dGTP, dTTP each, 4 mM MgCl$_2$) was added (final reaction volume 12.5 µL). A 2 µL aliquot was withdrawn at different time points, deproteinized by treatment with Proteinase K (0.5 mg/mL) and SDS (0.5% final) at 37°C for 15 min and subjected to 1.0% agarose gel electrophoresis in TAE buffer.

2.10 Second-end capture with DNA synthesis using an oligonucleotide D-loop

Second-end capture after DNA synthesis utilizing oligonucleotides was accomplished using a protocol established by McIlwraith and West (2008). A synthetic D-loop structure is constructed to have a short ssDNA region (> 29 bases). The third oligonucleotide, complementary to the ssDNA region, is annealed between the D-loop. DNA synthesis from the short oligonucleotide (29 bases) extends the D-loop further, providing ssDNA for a second overhang substrate to anneal. The synthetic D-loop substrate was constructed by annealing unlabeled OL3, OL4 and OL5. The synthetic overhang substrate was constructed by annealing $^{32}$P-labeled OL1 and unlabeled OL2. The overhang substrate is complementary to a short region on the
synthetic D-loop structure.

hPol η (0.08 µM) was incubated in the presence of either hSSB1 (0.27µM) or hSSB2 (0.18µM) with the synthetic D-loop structure and the 32P-labeled overhang substrate for 30 min at 37°C in buffer I (50 mM Tris–HCl pH 7.4, 1 mM DTT, 4 mM MgCl2, 0.125 mM dATP, dCTP, dGTP, dTTP each). The reactions were deproteinized at 37°C for 15 min and subjected to 8% native polyacrylamide gel electrophoresis in TAE buffer.

Table 2.2 Oligonucleotide sequences used to construct the synthetic D-loop and overhang.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OL1</td>
<td>CCGTCGCATGACGCTGCCGAATTCTACC</td>
</tr>
<tr>
<td>OL2</td>
<td>AGCGTCATGCGACGG</td>
</tr>
<tr>
<td>OL3</td>
<td>GCCAGGGACGGGTTGAAACCTGCAAGTGGGGCGGCTGCTCATCGTAGGTAGTAGTATCGACCTATTTGGTAGAATTCGCGCAGCGTCATGCGACGG</td>
</tr>
<tr>
<td>OL4</td>
<td>GCCGTCGCATGACGCTGCCGAATTCTACCAGCTACTAGGGTGCCCTTGCTAGGACATCTTTGCCCACCTGCAAGGTTCAACCCCAGTCC</td>
</tr>
<tr>
<td>OL5</td>
<td>AAGATGTCTAGCAAGGCACCCTAGTAGC</td>
</tr>
</tbody>
</table>

2.11 D-loop extension with second-end capture

Unlabeled D-loop substrate (OL90, 2.5 µM nucleotides) was incubated with 1.5 µM hSSB1 or 0.8 µM hSSB2 in Buffer C for 10 min at 37°C before the addition of
pBluescript SK replicative form I (35 µM base pairs). After an additional incubation at 37°C for 10 min, hPol η (0.25 µM) and 1.5 µL Buffer H was added to the reaction and further incubated at 37°C for 30 min. To detect second-end capture after DNA extension, 1 µL 32P-OL80 (2.5 µM nucleotides) was added to the reaction (final reaction volume 12.5 µL). The reactions were deproteinized at the indicated time points by treatment with Proteinase K (0.5 mg/mL) and SDS (0.8% final) at 37°C for 15 min and subjected to 1.0% agarose gel electrophoresis in TAE buffer.

3. Results

3.1 Phylogenetic Analysis confirms hSSB1 and hSSB2 are related to archaeal SSBs

The topology of our major tree (Appendix A, Figure A.1) indicates the possibility of major divergence events, leading to an ancestral separation between Archaea and Eubacteria. Our analysis of the tree suggests that SSBs, in a similar way to other components in Eukaryotes, could have been vertically inherited from an unidentified ancestral archaeon (Williams et al., 2012). The separation of the branches and the distribution of Eukaryotes within an Archaeal clade lends support to the eocyte hypothesis (Cox et al., 2008). Three diverse eukaryotic sequences (GenBank Accessions: CCA17796.1, EPS58601.1 and XP 002972708.1) with an OB fold domain similar to the archaeon Sulfolobus solfataricus (GenBank Accession: AAK42515) were identified. These were grouped within one of the Archaeal clades, further supporting the possibility that eukaryotic SSBs might have originated from a common Archaeal ancestor.
Further inspection of the major tree also reveals a major separation within Eukaryotes. In the major tree, it is possible to observe a divergence between the higher eukaryotic SSB1 and SSB2. Our data suggests that this differentiation might be a significant evolutionary event due to the prevalence of members of the Kingdom Animalia within the SSB1 and SSB2 clades. These findings were summarized in a simplified version tree (Figure 2.3), with topology that supports the hypothesis of SSB1 and SSB2 emerging as the result of a divergence within an ancestral eukaryotic SSB protein, thus giving rise to both subunits. The species displayed in the simplified tree are listed in Table 2.3.

![Figure 2.1 Phylogenetic analysis of hSSB1 and hSSB2.](image)

A simplified version of the phylogenetic analysis, indicating a major divergence event between higher eukaryotes and an ancestral eukaryotic SSB protein. The phylogeny includes representatives from the three domains of life where these proteins have been identified. Different taxonomic groups were identified by colors (hSSB1: blue, hSSB2: green, Archaea: red). The outgroup, Homo sapiens RPA 70 kDa (P35244), is black. Accession numbers are listed for each protein.
Table 2.3 Species used to generate the simplified version of the likelihood phylogenetic tree. Species name and respective GI reference number listed for each species. Different taxonomic groups were identified by colors (hSSB1: blue, hSSB2: green, Archaea: red).

<table>
<thead>
<tr>
<th>Species</th>
<th>GI Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SSB1</strong></td>
<td></td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>119617318</td>
</tr>
<tr>
<td>Condylyra cristata</td>
<td>507977215</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>348605242</td>
</tr>
<tr>
<td>Chrysemys picta bellii</td>
<td>530649259</td>
</tr>
<tr>
<td><strong>SSB2</strong></td>
<td></td>
</tr>
<tr>
<td>Monodelphis domestica</td>
<td>126326445</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>74195597</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>149046191</td>
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<tr>
<td>Mus musculus</td>
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<tr>
<td><strong>Archaea SSB</strong></td>
<td></td>
</tr>
<tr>
<td>Acidianus hospitalis W1</td>
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</tr>
<tr>
<td>Metallosphaera sedula DSM</td>
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</tr>
<tr>
<td>Sulfolobus solfataricus</td>
<td>15899120</td>
</tr>
<tr>
<td>Sulfolobus solfataricus</td>
<td>33357645</td>
</tr>
</tbody>
</table>

The bootstrap values in our major phylogenetic analysis might suggest that our proposed findings could result from methodological error. However, when we analyzed the three different protein trees generated under different substitution models, we were able to determine conservation in the topologies and distribution of species across the clades. The lower bootstrap values identified in some nodes could be attributed to the high level of divergence amongst the sequences.
3.2 Purification of hSSB1 and hSSB2

Both hSSB1 and hSSB2 were purified following the same protocol (Figure 2.1A Panel I). A C-terminal 6X-Histidine tag was utilized to aid in the purification of hSSB1 and hSSB2. hSSB1 (Figure 2.1A Panel II, lane 1) and hSSB2 (Figure 2.1A Panel II, lane 2) were determined to electrophoretically pure after Coomassie staining.

![Purification Scheme](image)

**Figure 2.2 (A.) Purification of hSSB1 and hSSB2.** Purification scheme for hSSB1 and hSSB2 (panel I). Purified hSSB1-(HIS)_6 (lane 1, panel II) and purified hSSB2-(HIS)_6 (lane 2, panel II).

3.3 hSSB1 and hSSB2 stimulate annealing of complementary ssDNA

RPA has been reported to stimulate ssDNA annealing in a concentration dependent manner; however, RPA requires secondary structure in ssDNA to facilitate annealing (Bartos et al., 2008). Secondary structure-free ssDNA, such as the oligonucleotides used in this study, can anneal independently over time (Figure 2.2B, panel I). In the absence of structure, RPA does not promote annealing. In fact, RPA binds the ssDNA and efficiently inhibits annealing. Both hSSB1 and hSSB2 would be expected to also inhibit ssDNA annealing, similar to RPA. We therefore tested the effect
of hSSB1 and hSSB2 on the rate of annealing ssDNA using radiolabeled oligonucleotides.

Unexpectedly, hSSB1 and hSSB2 did not inhibit the annealing of the ssDNA but rather accelerated the annealing of complementary DNA significantly (Fig 2.2B, panel I and II). Under the conditions used in this study, no secondary structure was present in the ssDNA, and RPA did not anneal the oligonucleotides (Figure 2.2B, panel IV). The scRad52 mediator has the ability to anneal ssDNA in the presence or absence of RPA (Sugiyama et al., 1998). We therefore utilized Rad52 as control in the ssDNA annealing assay.

Figure 2.3 hSSB1 and hSSB2 can anneal complementary ssDNA (A.) Schematic of the ssDNA annealing reaction. (B.) Unlabeled OL83-c (0.83 µM nucleotides) was incubated in absence (panel I) or in the presence of hSSB1 (0.27 µM, panel II), hSSB2 (0.18 µM, panel III), hRPA (0.18 µM, panel IV), scRad52 (0.34 µM, panel V) or scRPA (0.18 µM, panel VI) at 37°C for 5 min before the addition of 32P-OL83 (0.83 µM nucleotides). The reaction was quenched with excess unlabeled OL83-c and subjected to treatment with Proteinase K (0.5 mg/mL) and SDS (0.8% final) at the indicated times before separation on 12 % non-denaturing TAE polyacrylamide gels. (C.) The percentage of dsDNA annealed was quantified using ImageQuant (GE Healthcare) and plotted.
3.4 hSSB1 and hSSB2 can melt duplex DNA

Several SSB proteins, including RPA, *E. coli* SSB and *S. sulfolobus* SSB, have been shown to retain a conserved helix-destabilization activity (Bartos et al., 2008, Cubeddu and White, 2005, Delagoutte et al., 2011). It has also been reported that hSSB1 possesses dsDNA melting activity as well (Delagoutte et al., 2011). To examine duplex DNA melting activity of hSSB1 and hSSB2, we utilized an oligonucleotide-based assay modified from Cubeddu and White (2005). As previously reported, we were able to confirm dsDNA melting activity by hSSB1 and hSSB2 (Fig 2.4A panel II) (Delagoutte et al., 2011). Both hSSBs melted ~50% of the dsDNA in a time-dependent and ATP-independent manner (Fig 2.4A panel II).

Figure 2.4 hSSB1 and hSSB2 can melt duplex DNA (A.) Schematic of the homologous DNA pairing assay (panel I). Either 0.45 µM hSSB1 (panel II lanes 2-6) or 0.28 µM hSSB2 (panel II lanes 7-11) were incubated with unlabeled OL83 (10 µM nucleotides) at 37°C for 10 min before the addition of 32P-labeled OL83/OL83c (5 µM base pairs). Reactions were stopped at the indicated time points. Homologous DNA pairing activity was detected by the production of ssDNA. Lane 1 is a control with no protein. The percentage of ssDNA was quantified and graphed.
3.5 hSSB1 and hSSB2 can anneal ssDNA on plasmid-length substrates.

In addition to destabilizing dsDNA oligonucleotides, the archeal SSB from *Sulfolobus* was shown to efficiently melt supercoiled plasmid-length dsDNA (Cubeddu and White, 2005). To a lesser extent, RPA can also initiate unwinding of long dsDNA molecules but requires either A-T rich regions or assistance by a topoisomerase to relieve topological stress (Treuner *et al.*, 1998). Based on the strand annealing and duplex melting activity of the hSSBs, we examined the hSSBs activity using supercoiled plasmid length DNA substrates in a D-loop formation assay (Figure 2.5A panel I).

Surprisingly, both hSSB1 and hSSB2 were able to form a D-loop structure by destabilizing the supercoiled dsDNA molecule and annealing the complementary oligonucleotide (Fig 2.5A panel II). However, this activity is conceivably due to the DNA melting, strand annealing activity of the hSSBs and conservation of activity from the *Sulfolobus* SSB. The D-loop structure is formed quickly in an ATP-independent reaction and remains stable over time. Additionally, the slower migrating D-loop was persistent after treatment with Proteinase K and SDS, indicating the joint molecule was not product of protein-DNA aggregation.
Figure 2.5 hSSB1 and hSSB2 D-loop formation. (A.) Panel I is a diagram of the D-loop reaction. Either 1.5 µM hSSB1 (panel II lanes 2-6) or 0.8 µM hSSB2 (panel II lanes 7-11) was incubated with \(^{32}\)P-labeled OL90 (2.5 µM nucleotides) prior to the addition of supercoiled pBluescript SK replicative form I (35 µM base pairs). Times points were taken as indicated, and the percentage of D-loop formation was graphed below.

3.6 Second-end capture after D-loop formation

We next hypothesized that the hSSBs may be able to capture a second DNA molecule after D-loop formation. Second-end capture occurs in HR during post-synapsis, when the homologous ssDNA anneals to the displaced DNA at the D-loop. To detect second-end capture \textit{in vitro}, we followed the protocol from Nimonkar \textit{et al.} (2009). D-loop formation was first completed by hSSB1 and hSSB2 by annealing of the \(^{32}\)P-OL90 to the plasmid DNA (Figure 2.6A panel II, lanes 2 and 6). Second-end capture is
completed after binding of the second-end oligonucleotide and detected after restriction enzyme digestion. If the second DNA molecule was annealed, stabilization of the duplex D-loop structure occurs and is visible by the slower migrating DNA band. In contrast, the joint molecule rapidly dissociates after digestion due to the release of topological restraint of the plasmid DNA if the complementary ssDNA molecule is not annealed (Radding et al., 1977) (Figure 2.6A panel I, panel II lanes 5 and 9). Both hSSB1 and hSSB2 have the ability to capture a second complementary DNA molecule after D-loop formation (Figure 2.6A panel II, lanes 3 and 7).
Figure 2.6  hSSB1 and hSSB2 can capture second-end DNA. (A.) Schematic of the second-end capture assay (panel I). The D-loop structure was by formed by hSSB1 or hSSB2 after incubation with unlabeled OL90 (2.5 µM nucleotides) and pBluescript DNA (35 µM base pairs). Second-end capture was initiated by the addition of the second 32P-labeled DNA molecule (OL80). The reactions were digested with EcoR1 for 45 min at 37°C to detect second-end capture. Both hSSB1 (panel II lane 3) and hSSB2 (panel II lane 7) were able to anneal the complementary second DNA molecule to the D-loop structure but not when presented with heterologous DNA (panel II lanes 5 and 9). Lanes 2, 4, 6 and 8 (panel II) showed D-loop formation prior to digestion. Lanes 1 and 5 (panel II) were a control with no protein.

3.7 hPolymerase η physically interacts with hSSB1 and hSSB2

In DSB repair, polymerases are required for DNA synthesis during the final steps, essentially extending the D-loop formed between homologous chromosomes (Sebesta et al., 2013). Human Pol η has been implicated in DSB repair and was shown to preferentially bind D-loop structures (Kawamoto et al., 2005, Mcllwraith et al., 2005).
We were interested in whether hPol η might function cooperatively with hSSB1 and hSSB2.

To determine if the hSSBs physically interacted with hPol η in vitro, we utilized purified hPol η (Fig 2.7A lane 2) in an affinity pull-down assay. Since all three proteins (hSSB1, hSSB2 and hPol η) possessed a 6X-Histidine tag, we conjugated hSSB1 and hSSB2 to Affi-Gel beads. We showed that hPol η was retained on the Affi-hSSB1 and Affi-hSSB2 beads, indicating hPol η physically interacts with Affi-hSSB1 (Fig 2.7B lane 3) and Affi-hSSB2 (Fig 2.7B lane 6) but not Affi-BSA (Fig 2.7B lane 9).

Figure 2.7 Physical interaction of hSSBs with Pol η (A.) Purification scheme for hPol η (panel I). Lane 1 is low molecular weight standards (panel II). Lane 2 is purified hPol η- (HIS)_6. (B.) hPol η was incubated with Affi-hSSB1 (lanes 1-3), Affi-hSSB2 (lanes 4-6), or Affi-BSA (lanes 7-9) for 30 min at 4°C. The supernatant (S), wash (W) and eluate (E) were separated on a 12% SDS-PAGE gel and stained with Coomassie Blue. Pol η physically interacted with Affi-hSSB1 and Affi-hSSB2 (lanes 3 and 6).
3.8 hPol η extends D-loop substrates formed by hSSB1 and hSSB2

In addition to preferential binding, hPol η was previously shown to synthesize DNA from D-loop structures formed by either RAD51 or scRad52 (McIlwraith and West, 2008, Sebesta et al., 2013, Sneeden et al., 2013). Based on previous reports describing hPol η synthesis on D-loop structures and the physical interaction we observed between the hSSBs and hPol η, we speculated that hPol η might be able to synthesize from a hSSB-formed D-loop. Surprisingly, hPol η was able to extend from the joint molecules formed by hSSB1 and hSSB2, as evidenced by the slower migrating DNA (Figure 2.8A, panel II lanes 2-5 and 6-9). Extension of the DNA did not occur when hPol η, hSSB1 or hSSB2 was incorporated individually (Figure 2.8A, panel III lanes 1-3) or in the absence of dNTPs (Figure 2.8A, panel III lanes 4-8).
3.9 hSSB1 and hSSB2 stimulate hPol η DNA synthesis from an oligonucleotide D-loop structure

To further characterize the functional interaction between the hSSBs and hPol η, we constructed a synthetic D-loop structure to simulate second-end capture after DNA extension, as described in McIlwraith and West (2008). The synthetic D-loop contained a short oligonucleotide annealed to the displaced DNA, serving as the site for DNA extension. A radiolabeled overhang substrate complementary to one strand of the
synthetic D-loop would be annealed if DNA extension occurred, producing a significantly slower migrating DNA molecule.

Using this method, we were able to demonstrate that hSSB1 and hSSB2 anneal complementary oligonucleotides, which were formed after DNA synthesis by hPol η (Figure 2.9A lanes 5-8 and 9-12). hPol η has previously been shown to promote a slight amount of extension and annealing independently (McIlwraith and West, 2008). We also observed a lesser amount of final product formed by hPol η alone compared to the stimulation from hSSB1 and hSSB2 (Figure 2.9A lanes 1-4).
Figure 2.9 hSSB1 and hSSB2 stimulates Pol η extension from an oligonucleotide D-loop structure.

(A.) hPol η (0.08 µM) was incubated either alone (lanes 1-4, panel I) or in the presence of 0.27 µM hSSB1 (lanes 5-8, panel I) or 0.18 µM hSSB2 (lanes 9-12) for 30 min at 37°C. The reaction products were deproteinized separated using electrophoresis on an 8% native polyacrylamide gel. Size markers on the left indicate the possible DNA combinations while markers on the right indicate the starting 32P-labeled overhang and the final extended D-loop structure after second-end capture and extension. (B.) Percent of extended D-loop product was quantified and graphed at the indicated times points.
3.10 Second-end capture after D-loop extension by hPol η

After detecting DNA extension and second-end capture using oligonucleotides, we modified a protocol from Mazloum and Holloman (2009) to confirm synthesis-dependent second-end capture using plasmid-length substrates. Briefly, the D-loop was formed by either hSSB1 or hSSB2 using unlabeled OL90 and pBluescript SK replicative form I DNA. Evidence of second-end capture was visualized using $^{32}$P-labeled OL80, which is complementary to pBluescript 50 bases downstream from the OL90 sequence. However, it is important to note that extension of the D-loop by hPol η is required to allow the $^{32}$P-OL80 to anneal.

Both hSSB1 and hSSB2 were able to promote second-end DNA capture after extension by hPol η (Figure 2.10A, panel II lanes 5-8 and 9-12). D-loop and extended D-loop products (formed by hSSB1) were used as a marker for the second-end capture after extension (Figure 2.10A, lanes 2-3 panel II). Second-end capture was not visualized when hPol η, hSSB1 or hSSB2 were included individually (Figure 2.10B, lanes 2-4) or in the absence of dNTPs (Figure 2.10B, lanes 5-7), providing support that extension of the D-loop was required before the second oligonucleotide could be annealed. The slowest migrating bands (highest extended D-loop products) are thought to be produced by a dimer between two plasmid molecules after extension (Mazloum and Holloman et al., 2009). Extension of the plasmid DNA releases topological restraint and may provide a free 5' end that is subsequently annealed to the second plasmid molecule, leading to the higher D-loop band.
Figure 2.10 hPol η extended D-loop with second-end capture by hSSB1 and hSSB2. (A.) Schematic of D-loop extension and second-end capture reaction (Panel I). Lane 1 is a control with no protein (panel II). hSSB1 (1.5 µM, lanes 2-3 and 5-8) or hSSB2 (0.8 µM, lanes 9-12) were incubated with unlabeled OL90 before the addition of pBluescript SK replicative form I (35 µM base pairs) for 10 min at 37°C. Pol η (0.25 µM) was added and further incubated for 30 min at 37°C before the addition radiolabeled OL80. The reaction was deproteinized at the indicated times by treatment with Proteinase K (0.5 mg/mL) and SDS (0.8% final) for 15 min at 37°C. The reaction products were separated on a 1% native agarose gel and
dried before imaging. Second-end capture after DNA synthesis is visualized by the slower migrating D-loop. (B.) A series of controls. Lane 1 is a control with no protein. Either hSSB1 (lanes 2 and 5), hSSB2 (lanes 3 and 6) or Pol η (lanes 4 and 7) were incubated individually in the presence (lanes 1-4) or absence (lanes 5-7) of dNTPs (0.125 µM each).

4. Discussion

SSB proteins are found in all cellular organisms and are essential to the viability of an organism (Wold, 1997, Wang et al., 2005, Richard et al., 2008). Although all SSB proteins share some conservation, such as OB-folds, the function of these genes are diverse. Previous reports of the hSSBs have suggested a role in HR through ATM-activation, localization of Rad51 to DSBs, stimulation of the MRN complex in DSB processing and overall genomic stability (Richard et al., 2008, Huang et al., 2009, Li et al., 2009, Skaar et al., 2009, Feldhahn et al., 2012, Shi et al., 2013).

Here, we have identified several surprising activities of hSSB1 and hSSB2. Both hSSBs have an efficient ssDNA annealing activity and, similar to other SSBs, the hSSBs retain the ability to melt duplex DNA in vitro. The hSSBs rapidly localizes to the site of DSBs as an early response, when dsDNA has yet to be resected (Richard et al., 2008). Duplex DNA melting by SSBs could be useful in unwinding the dsDNA present at the break. Additionally, helix destabilization by SSBs can be utilized during the restart of a stalled replication fork (Vassin et al., 2009).

We also demonstrated that hSSB1 and hSSB2 can form a D-loop structure on plasmid DNA, and the D-loop structure is formed quickly in an ATP-independent manner and remains stable over time. Evidence supporting the formation of D-loop structure by the hSSBs include stability after protein degradation, dissolution of the D-loop after
restriction enzyme digestion, and stabilization by the capture of a second homologous oligonucleotide. D-loop formation by the hSSBs may be useful during the repair of stalled replication forks. Our results provide a possible mechanism in support of recent genetic studies in mice that implicate the hSSBS in replication-mediated DNA repair (Feldhahn et al., 2012, Shi et al., 2013, Gu et al., 2013).

Recently, RPA has been shown to stimulate polymerase extension from a D-loop structure (Sneeden et al., 2013). In contrast to a previously documented inhibitory role, RPA significantly increased the efficiency of D-loop extension by hPol δ (Li et al., 2009, Sneeden et al., 2013). RPA stabilizes D-loop formation through binding of the displaced strand, but the role of RPA binding to the template strand was not established (Eggler et al., 2002). Polymerases often stall during synthesis due to topological constraints induced by supercoiling or at collapsed replication forks. It appears that RPA may assist the polymerases by relieving topological stress and preventing stalling (Sneeden et al., 2013). It is important to note that RPA is assisted by topoisomerases to remove the supercoiling during at least the first 50 bases. However, RPA seems sufficient to maintain the linear structure further downstream (Sneeden et al., 2013). It is possible that hSSB1 and hSSB2, like RPA, stimulates polymerase activity by removing secondary structure.

The hSSBs are not required for the progression of normal replication; however, the hSSBs have recently been shown to localize to the stalled replication forks after DNA damage (Richard et al., 2008, Bolderson et al., 2014). Furthermore, in the absence of hSSB1, DNA DSBs accumulate rapidly after induced replication fork stalling (Bolderson
Here, we support the role of the hSSBs in the repair of stalled replication forks by demonstration of physical interaction with hPol η in addition to extension of hSSB-formed D-loops by hPol η. The amount of DNA extension was not determined here. However, extension by hPol η was at least 50 bases, as the radiolabeled primer used for second end capture would only be complementary after extension 50 bases downstream. Interestingly, RPA requires topoisomerase assistance to stimulate polymerase activity during the initiation of replication (Sneeden et al., 2013). Stalled replication forks structurally resemble D-loop structures formed in HR. It is likely that the hSSBs, like RPA, relieve topological stress formed during the unwinding of replication fork.

The proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) have been shown to significantly stimulate polymerase activity (Li et al., 2009, Overmeer et al., 2010). Interaction between the hSSBs with PCNA or RFC has not yet been demonstrated; however, it would be helpful to examine PCNA and RFC with the hSSBs to further characterize the role of the hSSBs in replication repair. Polymerase δ has been suggested as the prominent polymerase in the HR pathway and also functions during lagging-strand DNA synthesis (Maloisel et al., 2008). As seen with hPol η, hPol δ may also functionally interact with the hSSBs. Activity of the hSSBs with hPol δ would further support a role in the repair of stalled replication forks.

Both hSSB1 and hSSB2 form a heterotrimeric complex with INTS3 and hSSBIP1 (Huang et al., 2009, Li et al., 2009). INTS3 is part of the integrator complex, which interacts with RNA polymerase (Baillat et al., 2005). In the hSSB complex, INTS3
functions as a scaffolding protein to the hSSBs and regulates localization to DSBs (Skaar et al., 2009). The role INTS3 and hSSBIP1 play on the hSSBs polymerase stimulation would be very interesting to examine.

References


CHAPTER 3

BIOCHEMICAL CHARACTERIZATION OF HUMAN SINGLE-STRAND DNA BINDING PROTEINS

Abstract

In homologous recombination (HR), human single-strand DNA binding (hSSB) proteins are rapidly recruited to DNA double-strand breaks (DSBs), where hSSB1 and hSSB2 have diverse roles. In this report, we have extensively characterized the stimulation effect of hSSB1 on RAD51-mediated D-loop and concluded that hSSB1 and hSSB2 can independently form D-loop structures, in the absence of RAD51. We have examined the effect of CaCl$_2$ and MgCl$_2$ on D-loop formation and demonstrated DNA binding activity of hSSB2. Finally, we purified INTS3 and hSSBIP1 and confirmed physical interaction as the hSSB heterotrimeric complex.

1. Introduction

The human single-strand DNA binding proteins, hSSB1 and hSSB2, were shown to play a role in homologous recombination (HR) and were activated in response to DSBs (Richard et al., 2008, Huang et al., 2009, Li et al., 2009). Loss of either hSSB results in checkpoint defects, increased sensitivity to ionizing radiation, and impaired HR (Richard et al., 2008). Furthermore, recent reports indicate that the hSSBs function in replication-mediated DNA repair (Feldhahn et al., 2012, Gu et al., 2013, Bolderson et al., 2014). Taken together, hSSB1 and hSSB2 are required to maintain genomic integrity.

Like the hSSBs, Replication protein A (RPA) was shown to function in multiple
DNA repair pathways, including HR (Wold, 1997, Sugiyama et al., 1997, Haruta et al., 2006, Sung, 1997a, Yuzhahov et al., 1999). However, RPA and the hSSBs do not have overlapping functions. In response to DNA double-strand breaks (DSBs), both RPA and hSSB1 rapidly localize to DSB foci but co-localization on DNA is not observed (Wold, 1997, Richard et al., 2008). In fact, RPA co-localizes at RAD51 foci while hSSB1 does not (Golub et al., 1998, Raderschall et al., 1999, Richard et al., 2008). Furthermore, hSSB1 localization is not regulated by cell-cycle phase while RPA regulates S-phase progression, essentially delaying the cell-cycle pending DSB repair (Richard et al., 2008, Wold, 1997).

Like RPA, hSSB1 and hSSB2 form heterotrimeric complexes, designated as the sensor of single-stranded DNA (SOSS), along with Integrator subunit 3 (INTS3) and human single-strand binding interacting protein 1 (hSSBIP1) (Huang et al., 2009, Li et al., 2009, Zhang et al., 2009). INTS3 also functions in small nuclear RNA processing as part of the Integrator complex while hSSBIP1 is specific to the SOSS complex (Baillat et al., 2005). INTS3 has several significant functions in the SOSS complex, including recruiting hSSB1/2 and hSSBIP1 to the site of DSBs and serving as a scaffold protein for hSSB1/2 and hSSBIP1 (Li et al., 2009, Skaar et al., 2009, Huang et al., 2010). The SOSS complex also stimulates both the MRN complex and Exo1, indicating an early role in HR (Richard et al., 2011, Yang et al., 2013). Initially, hSSB1 and hSSB2 appeared to regulate ATM activation and checkpoint signaling, but further analysis suggested that INTS3 may have a more prominent role with ATM, as in vivo data does not support
hSSB1 or hSSB2 involvement (Richard et al., 2008, Feldhahn et al., 2012, Shi et al., 2013).

Recently, a second integrator subunit (INTS6) was identified as part of the SOSS complex (Zhang et al., 2013). Like INTS3, INTS6 is also a component of the integrator complex, in addition to localizing to DSBs as a subunit of the SOSS complex (Baillat et al., 2005, Zhang et al., 2013). In the previous chapter, a surprising ability of both hSSB1 and hSSB2 to anneal complementary ssDNA substrates, 'melt' dsDNA and produce D-loop structures in an ATP-independent manner was demonstrated. Furthermore, a novel role for the hSSBs through physical and functional interaction with the human polymerase η (Eta) was identified.

Here, the biochemical properties of the hSSB1 and hSSB2 D-loop activity and specifically, the effect of MgCl₂ and CaCl₂ on the hSSBs, in the presence and absence of RAD51 were examined. Finally, INTS3 and hSSBIP1, components of the SOSS complex, were purified and physical interaction between the proteins was confirmed.

2. Materials and Methods

2.1 Protein Purification

Unless otherwise stated, all protein purifications were treated in the same manner regarding the following steps. All bacterial expression plasmids were transformed into E. coli Rosetta (DE3) cells and grown at 37°C to an \( A_{600} \) of ~ 1.0 before the addition of 0.4 mM IPTG. After ~ 20 hr incubation at 16°C, the cells were harvested by centrifugation using a Beckman JLA 16.250 rotor at 4500 g at 4°C.
2.1a hSSB1 and hSSB2 purification protocol

hSSB1 and hSSB2 were purified following the same protocol. Both were expressed and harvested following standard procedure (Section 2.1). 60 g of cell paste was resuspended in 300 mL Buffer A (50 mM Tris pH 7.5, 1 mM EDTA, 10% sucrose, 0.01% Igepal, 1 mM β-mercaptoethanol, 0.1 mg/mL lysozyme, 1 mM benzamidine, 1 mM PMSF, and protease inhibitors: aprotinin, chymostatin, leupeptin, and pepstatin A at a final concentration of 5 µg/mL) with 150 mM KCl and lysed by sonication at a constant output at of 6 for 3 times for 30 second cycles. The cell extract was clarified by ultracentrifugation in a Beckman Ti.45 rotor at 100,000 g for 90 min at 4°C. All purification steps after lysis were carried out at 4°C. The clarified supernatant was diluted 1:3 in Buffer B (20 mM KH₂PO₄ pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, and 1 mM β-mercaptoethanol) containing 100 mM KCl and loaded onto a 65 mL Q-sepharose column. The protein was fractionated in Buffer B with a gradient containing 150 – 800 mM KCl. Fractions containing hSSB1 were incubated overnight with 1 mL Ni-NTA Sepharose 6 Fast Flow beads (GE Healthcare) in Buffer B with 300 mM KCl and 40 mM imidazole. After washing the beads with 10 column volumes of Buffer B containing 1M KCl and 40 mM imidazole, the beads were washed with an additional 10 column volumes of Buffer B with 300 mM KCl and 40 mM imidazole. The protein was eluted in Buffer B containing 300 mM KCl and 500 mM imidazole. Eluted fractions were diluted in Buffer B containing 100 mM KCl and loaded onto a 1 mL Macro Hydroxyapatite column (Bio-Rad). The protein was fractionated with Buffer C (20 mM
KH$_2$PO$_4$ pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, and 1 mM dithiothreitol) containing 300 mM KH$_2$PO$_4$ and 100 mM KCl. Peak fractions were diluted 1:4 with Buffer B and loaded onto a 1 mL Source S column (GE Healthcare). The protein was fractionated in Buffer B containing 100 mM KCl. Peak fractions were pooled and concentrated before aliquots were stored at -80°C.

2.1b hRAD51 Expression and Purification

The RAD51(HIS)$_6$ expression plasmid was transformed in E. coli BLR(DE3) strain cells and grown as stated in section 2.1. After harvest, 60 g cell paste was resuspended in Buffer D (50 mM Tris pH 7.4, 10% sucrose, 150 mM NaSO$_4$, 0.01% Igepal, 1 mM β-mercaptoethanol, 0.1 mg/mL lysozyme, 1 mM benzamidine, 1 mM PMSF, and protease inhibitors: aprotinin, chymostatin, leupeptin, and pepstatin A at a final concentration of 5 µg/mL) containing 600 mM NaCl before sonication at a constant output at of 6 (3 times for 30 seconds each). The cell extract was separated by ultracentrifugation in a Beckman Ti.45 rotor at 100,000 g for 90 min at 4°C. The clarified supernatant was diluted 1:5 in Buffer B and loaded onto a 65 mL Q Sepharose column (GE Healthcare). The protein was fractionated in Buffer B with a gradient containing 150 mM to 700 mM KCl. Peak fractions were pooled and incubated overnight with 1 mL Ni-NTA Sepharose 6 Fast Flow beads (GE Healthcare). The eluted protein was diluted 1:3 with Buffer B containing 150 mM KCl, loaded onto a 1 mL Macro Hydroxyapatite column (Bio-Rad) and fractionated in Buffer C with a gradient of 0 to 400 mM KH$_2$PO$_4$ and 150 mM KCl. Peak fractions were pooled, diluted 1:4 with Buffer C containing 100 mM KCl, and loaded onto a 1 mL Source S column (GE
Healthcare). The protein was eluted in Buffer C with a 30 mL gradient from 100 mM to 400 mM KCl. Peak fractions were concentrated to 10 mg/mL, and aliquots were stored at -80°C.

2.1c INTS3 Protein Purification

Clarified supernatant was obtained through resuspension of 60 g of cell paste in 300 mL Buffer A with 150 mM KCl and lysed by sonication at a constant output at of 6 for 3 times for 30 second cycles. The extract was clarified by ultracentrifugation in a Beckman Ti.45 rotor at 100,000 g for 90 min at 4°C. All purification steps after lysis were carried out at 4°C. The supernatant was diluted 1:3 in Buffer B containing 100 mM KCl and loaded onto a SP-sepharose column (GE Healthcare). The protein was fractionated in Buffer B with a gradient of 100 mM to 700 mM KCl. Peak fractions were incubated with 0.5 mL Ni-NTA Sepharose 6 Fast Flow beads (GE Healthcare) in Buffer B with 300 mM KCl and 40 mM imidazole overnight. The flow-through was collected, and the beads were washed with 5 mL of Buffer B containing 1 M KCl and 40 mM imidazole prior to a wash of 5 mL of Buffer B containing 300 mM KCl and 40 mM imidazole. Protein was eluted in 1.5 mL of Buffer B containing 300 mM KCl and 500 mM imidazole. Eluted fractions were then pooled and diluted 1:4 with Buffer C containing 150 mM KCl and loaded onto a 0.5 mL Source Q column (GE Healthcare). The column was washed with 5 mL of Buffer C containing 150 mM KCl and developed in a 15 mL gradient from 150 to 600 mM KCl in Buffer C. Peak fractions were diluted 1:2 with Buffer C containing 100 mM KCl and passed over a 0.5 mL Source S column (GE Healthcare). The column was washed with 5 mL Buffer C with 100 mM KCl and
fractionated with a 15 mL gradient of Buffer C containing 100 mM–400 mM KCl. Peak fractions (~200 mM KCl) were pooled and concentrated to 5 mg/mL in a Centricon-30 concentrator. Aliquots of the purified protein were stored at -80°C.

2.1d hSSBIP1 Cloning, Expression and Purification

The hSSBIP1 cDNA was PCR-amplified to generate a 5' (HIS)₆ tag (5' GGGA TCCTCCATGGGACACCATCACCATCACCATGGAGGAGCAGCAAACCTCTTCAG GACAA3'). The PCR product was inserted into the bacterial expression plasmid pGEX-6P-1 (GE Healthcare) vector by restriction enzyme digestion (BamH1) that contained a Precision protease cleavage site. The N-terminal GST-tag on hSSBIP1 was used to increase the protein solubility from cell lysate. The gene was sequenced to ensure no undesired mutations were present before the pGEX-(HIS)₆-hSSBIP1 expression vector was transformed into E. coli Rosetta (DE3) strain cells and grown at 37°C to an A₆₀₀ of ~1.0 prior to the addition of IPTG to 0.4 mM. 30 g of cell paste was resuspended in Buffer A containing 250 mM KCl and subjected to sonication 3 times at a constant output at of 4 for 30 seconds each. Clarified supernatant was obtained after ultracentrifugation at 100,000 g for 90 min in a Beckman Ti.45 rotor and was incubated overnight at 4°C with 0.5 mL Ni-NTA Sepharose 6 Fast Flow beads (GE Healthcare) in Buffer B with 300 mM KCl and 40 mM imidazole. The flow was collected prior to washing the beads with 5 mL Buffer B containing 1 M KCl and 40 mM imidazole followed by a 5 mL wash of Buffer B containing 300 mM KCl and 40 mM imidazole. The protein was eluted with Buffer B containing 300 mM KCl and 500 mM imidazole. The eluted protein was diluted 1:1 with Buffer B and incubated with Precision protease for 2 hrs at 4°C to cleave the N-terminal
GST-tag. The protein was diluted again 1:4 in Buffer B containing 300 mM KCl before an overnight incubation at 4°C with 0.5 mL glutathione sepharose beads (GE Healthcare) to remove the Precision protease. Flow-through was collected and incubated a second time with Ni-NTA beads for 2 hrs at 4°C. The protein was eluted following the same protocol as above and concentrated to 3.5 mg/mL in a Centricon-3 concentrator. Aliquots of the purified protein were stored at -80°C.

2.2 φX174 DNA electrophoretic mobility shift assay

Increasing amounts of hSSB1 or hSSB2 (as indicated) were incubated with either ssDNA (φX174 viral (+) strand, 30 μM nucleotides) or dsDNA (linearized φX174 replicative form I, 15 μM base pairs) for 10 min at 37°C in Buffer D (20 mM Tris HCl pH 7.5, 50 mM KCl, 1 mM DTT). The final reaction volume was 12.5 μL. A control reaction was deproteinized by treatment with Proteinase K (0.5 mg/mL) and SDS (0.5% final) and incubated an additional 10 min at 37°C. The samples were resolved on 1.0% agarose gels and stained with ethidium bromide.

2.3a RAD51 D-loop Assay

The D-loop substrate (OL90, 5’AAATCAATCTAAAGTATATGAGTAAAC TTGGTCTGACGTTACCAATGCTTAATCAGTGAGGACCTATCTCAGCGATCT GTCTATT-3’) was radiolabeled with [γ-32P]-ATP using T4 polynucleotide kinase. Micro Bio-Spin 30 Columns (Bio-Rad) were utilized to remove unincorporated [γ-32P]ATP. The 32P-OL90 (2.5 μM nucleotides) was incubated with 1.0 μM RAD51 for 5 min at 37°C before the addition of increasing hSSB1 or hSSB2 in Buffer E (25 mM Tris-HCl,
pH 7.5, 1 mM dithiothreitol, 50 mM KCl, 2.0 mM ATP, 20 mM creatine phosphate, 20 µg/ml creatine kinase) for 3 min. CaCl$_2$ was added to the reaction at the indicated amounts (either 5 mM, 0.5 mM or 0 mM) for an additional 2 min. The reaction was initiated by addition of pBluescript SK replicative form I (35 µM base pairs) and further incubated for 10 min (final reaction volume 12.5 µL). The reaction was deproteinized by treatment with Proteinase K (0.5 mg/mL) and SDS (0.5% final) at 37°C for 10 min and subjected to 0.9% agarose gel electrophoresis in TAE buffer. The gels were dried, analyzed with a phosphorimager and quantified using ImageQuant software (GE Healthcare). All subsequent gels were analyzed in the same manner unless otherwise stated. RAD51 D-loop reactions containing MgCl$_2$ were completed following the same procedure with the following exception: 2.4 mM MgCl$_2$ was included in Buffer E.

2.3b hSSB1 and hSSB2 D-loop titrations

Either hSSB1 (1.5 µM) or hSSB2 (0.8 µM) was incubated with $^{32}$P-OL90 (2.5 µM nucleotides) in Buffer F (25 mM Tris-HCl pH 7.5, 1 mM dithiothreitol, 50 mM KCl) for 5 min at 37°C before the addition of increasing CaCl$_2$ or MgCl$_2$. After an additional 5 min incubation, the reaction was initiated by the addition of pBluescript SK replicative form I (35 µM base pairs) and further incubated for 10 min. The reaction was then deproteinized by treatment with Proteinase K (0.5 mg/mL) and SDS (0.5% final) at 37°C for 10 min. The reaction products were subjected to 0.9% agarose gel electrophoresis in TAE buffer.
2.4 Affinity Pull-down assay

To examine physical interactions between the complex proteins, hSSB1/2, INTS3 and hSSBIP1, Affi-Gel matrix beads were utilized. Either hSSB1 (10 mg), hSSB2 (10 mg) or BSA (10 mg) were immobilized on 0.5 mL of Affi-Gel matrix beads (Bio-Rad) each at 4°C in Buffer G (100 mM MOPS pH 7.5) for 4 hrs and stored in Buffer H (50 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 30% glycerol) at -20°C. Before use, the Affi-gel matrices were washed with Buffer I (20 mM KH₂PO₄ pH 7.5, 1 mM DTT, 10% glycerol) containing 100 mM KCl. INTS3 (5µg) or hSSBIP1 (5µg) was added to the indicated Affi-gel complex in Buffer H to a final volume 30 µL, and the samples were subjected to gentle agitation at 4°C for 30 min. The supernatant was collected prior to 3 washes with Buffer H containing 100 mM KCl. Each sample received 30 µL of 2x SDS dye before incubation at 95°C for 1 min. The supernatant, wash and bead samples containing INTS3 were loaded on 10% SDS-PAGE while samples containing hSSBIP1 were loaded on 18% SDS-PAGE.

To examine complex formation between all 3 proteins, the S-protein on INTS3 was exploited. The S-protein resin was washed 3 times with Buffer I containing 100 mM KCl before the addition of hSSB1 (5µg), hSSBIP1 (5µg) and INTS3 (5µg). Buffer I was added to reach a final reaction volume of 30 µL. The reaction was gently agitated at 4°C for 30 min. The supernatant, wash, and bead fractions were retrieved as described and subjected to 15% SDS-PAGE. All gels were stained with Coomassie Blue for visualization.
3. Results

3.1 hSSB2, like hSSB1, preferentially binds ssDNA.

The DNA binding activity of hSSB1 was previously shown (Richard et al., 2008). Here, we utilized an electrophoretic mobility shift assay (EMSA) with plasmid-length substrates, φX174 ssDNA and linearized φX174 RF dsDNA, to examine the DNA binding activity of the hSSBs. Increasing concentrations of either hSSB1 or hSSB2 were able to completely shift the ssDNA molecule but not the dsDNA molecule (Figure 3.1, Panels I – IV).

Figure 3.1 hSSB1 and hSSB2 bind ssDNA but not dsDNA. (A). Either hSSB1 or hSSB2 (All panels, Lane 2, 0.1 µM, lane 3, 0.25 µM, lane 4, 0.5 µM, lane 5, 1 µM) were incubated with φX174 ssDNA (30 µM nucleotides) (Panel I hSSB1, Panel III hSSB2) or linearized φX174 RF dsDNA (30 µM base pairs) (Panel II hSSB1, Panel IV hSSB2) at 37°C for 10 min. Reaction products were separated on 0.9 % agarose gels and stained with ethidium bromide. Lanes 1 contained no protein (NP) in all panels. Lanes 6 in all panels were controls treated with Proteinase K (PK) (0.5 mg/mL) and SDS (0.5% final) at 37°C for 10 min prior to gel electrophoresis.
3.2 Effect of CaCl$_2$ on the activity of hSSB1 and hSSB2 RAD51-mediated and independent D-loop formation

The RAD51 recombinase is responsible for D-loop formation during mitotic HR. The ability to stimulate RAD51-mediated D-loop activity is commonly used to identify proteins that function in HR. RPA has been shown to increase D-loop formation of RAD51 but importantly, only when RPA is included in the reaction after RAD51 filament formation (Sugiyama et al., 1997, Sung and Robberson, 1995). After RAD51-filament formation, RPA stimulates the recombinase by eliminating secondary structure from forming in the ssDNA and prevents reannealing by sequestering the displaced ssDNA (Yuzhahov et al., 1999). In 2008, Richard et al. reported that hSSB1 also retains the ability to stimulate RAD51-mediated D-loop. It was noted (but not shown) that when hSSB1 is included prior to RAD51-filament formation, RAD51 activity was inhibited.

In our efforts to characterize hSSB2, we first confirmed RAD51-mediated stimulation by hSSB1, using the same conditions as Richard et al., 2008. We indeed were able to replicate hSSB1 stimulation similar to a previous report (Figure 3.2B Panel I, lanes 2-5). However, when attempting to replicate inhibition of RAD51, we noticed that hSSB1, in the absence of RAD51, independently formed a D-loop structure (Figure 3.2B Panel I, lanes 6-8). Our initial thoughts were that the hSSB1 activity was an artifact of the in vitro conditions used to demonstrate RAD51 stimulation, specifically the CaCl$_2$ concentration. We therefore reduced or eliminated the CaCl$_2$ in the reaction; however, D-loop formation by hSSB1 significantly increased and the stimulatory effect of hSSB1 on RAD51 was no longer apparent (Figure 3.2B Panel I, lanes 10-16 and 18-24).
Accordingly, hSSB2 activity was, although slightly reduced, comparable to hSSB1 D-loop activity (Figure 3.2C Panel I, lanes 2-8, 10-16 and 18-24). We also examined RPA activity using the same conditions but did not observe stimulation or independent formation of D-loop products (Figure 3.2D Panel I, lanes 2-12, 14-24 and 26-36).
Figure 3.2 Effect of calcium on RAD51-mediated and hSSB D-loop. (A.) Schematic of D-loop reaction. RAD51 (1.0 µM) was incubated with \( ^{32}P \)-OL90 (2.5 µM nucleotides) for 5 min either alone (Lane 2) or in the presence of (B.) hSSB1 (2 µM, 4 µM and 8 µM), (C.) hSSB2 (2 µM, 4 µM and 8 µM) (D.) or RPA (0.1, 0.2, 0.4, 0.8 and 1.0 µM) prior to the addition of CaCl\(_2\) at the indicated concentrations and
further incubated for 2 min. Reactions were initiated by 1 µL of supercoiled pBluescript SK replicative form I (35 µM base pairs) for 10 min, deproteinized and separated on 0.9% agarose gels. Percentage of D-loop formation was graphed in panel II for each.

3.3 Effect of constant MgCl₂ on hSSB1 and hSSB2 RAD51-mediated and independent D-loop formation

MgCl₂ is often required during in vitro recombination assays as a cofactor for ATP-hydrolyzing enzymes. We therefore decided to examine the effect of MgCl₂ on RAD51 and hSSB D-loop activity. MgCl₂ remained constant while CaCl₂ was included as in section 3.2. Although D-loop formation was slightly reduced in the presence of both CaCl₂ and MgCl₂, both hSSB1 and hSSB2 still independently formed D-loop structures while RPA did not (Figure 3.3 Panels I, II and III).
Figure 3.3 Effect of calcium and constant MgCl₂ on RAD51-mediated and hSSB D-loop. (A.)
Schematic of D-loop reaction. RAD51 (1.0 µM) was incubated in the presence of 2.4 µM MgCl₂ with 32P-OL90 (2.5 µM nucleotides) for 5 min either alone (Lane 2) or in the presence of (B.) hSSB1 (2 µM, 4 µM and 8 µM), (C.) hSSB2 (2 µM, 4 µM and 8 µM) (D.) or RPA (0.1, 0.2, 0.4, 0.8 and 1.0 µM) prior to the addition of CaCl₂ at the indicated concentrations and further incubated for 2 min. Reactions were initiated by 1 µL of supercoiled pBluescript SK replicative form I (35 µM base pairs) for 10 min, deproteinized and separated on 0.9% agarose gels. The percentage of D-loop formation was graphed in panel II for each.
3.4 CaCl$_2$ and MgCl$_2$ effect on hSSB1 and hSSB2 D-loop activity

In an effort to provide a more detailed picture of CaCl$_2$ and MgCl$_2$ concentrations on hSSB1 and hSSB2 D-loop activity, we titrated either CaCl$_2$ or MgCl$_2$ into the reaction. It is important to note that the percentage of D-loop formation by hSSB1 and hSSB2 are not comparable to those found in Figures 3.2 or 3.3, as the starting concentration of the hSSBs was optimized for D-loop formation prior to the titrations. The concentrations used in the RAD51-mediated D-loop assay were followed as reported in Richard et al., 2008. Both hSSB1 and hSSB2 are more capable of annealing the complementary D-loop substrate to the plasmid DNA in the absence of (or at low concentration of) either CaCl$_2$ or MgCl$_2$ (Figure 3.4A and B Panels I and II, lane 2). Surprisingly, high concentrations of CaCl$_2$ had an inhibitory effect on hSSB2 but not hSSB1 D-loop activity (Figure 3.4A Panel III). In contrast, the MgCl$_2$ concentration, although slight, decreased hSSB1 activity at a lower concentration compared to hSSB2 (Figure 3.4B Panel III).
Figure 3.4 Effect of CaCl\textsubscript{2} and MgCl\textsubscript{2} on hSSB1 and hSSB2 D-loop formation.

(A.) hSSB1 (1 µM) (Panel I) or hSSB2 (0.6 µM) (Panel II) was incubated with 32\textsuperscript{P}-OL90 (2.5 µM nucleotides) for 5 min in the presence of either increasing CaCl\textsubscript{2}. The reactions were initiated by 1 µL of supercoiled pBluescript SK replicative form I (35 µM base pairs) for 10 min. Reactions were deproteinized and separated on 1.0% agarose gels. The percentage of D-loop formation was graphed in Panel III for each.

(B.) Increasing MgCl\textsubscript{2} concentrations were included in hSSB1 (Panel I) and hSSB2 (Panel II) D-loop reaction. The methods were followed as in (A). Percentage of D-loop formation was graphed in Panel III.
3.5 Purified SOSS complex components, INTS3 and hSSBIP1

We purified both hSSBIP1 (Figure 3.5A Panel II) and INTS3 (Figure 3.5B Panel II) and examined physical interactions with hSSB1 and hSSB2. hSSBIP1 was unable to bind either hSSB1, hSSB2 or INTS3 (Figure 3.5C Panels I and III). However, INTS3 strongly interacted with both hSSB1 and hSSB2 individually (Figure 3.5C Panel IV) and as a heterotrimeric complex (Figure 3.5C Panel II). Using the purified recombinant proteins, we confirmed that INTS3 is the central component in the SOSS complex (Huang et al., 2009, Li et al., 2009, Zhang et al., 2009, Ren et al., 2014).
Figure 3.5 Purification and interactions of hSSBIP1-(HIS)$_6$ and INTS3-(HIS)$_6$. (A.) Purification scheme for hSSB1P1 (panel I) and purified hSSB1-(HIS)$_6$ protein (panel II). (B.) Purification scheme (Panel I) for INTS3-(HIS)$_6$ (panel II). (C.) Affinity Pull-downs of the hSSB complex proteins. hSSB1, hSSB2 and BSA were conjugated to Affi-gel matrix beads to identify physical interaction with hSSBIP1 and INTS3. hSSBIP1 interaction with either hSSB1 (Lanes 1-3), hSSB2 (Lanes 4-6) (Panel I) or INTS3 (Lanes 1-3) (Panel III) was examined individually. Complex interaction with INTS3, hSSBIP1 and hSSB1 (Lanes 1-3) was examined in Panel III. INTS3 interaction with either hSSB1 (Lanes 1-3) or hSSB2 (Lanes 4-6) was demonstrated in Panel IV. The supernatant (S), wash (W) and elution (E) were analyzed after electrophoresis using SDS-PAGE and stained with Coomassie Brilliant Blue.
4. Discussion

Single-strand DNA binding (SSB) proteins play a vital role in DNA repair and recombination (Wold, 1997, Richard et al., 2008, Souquet et al., 2013). Previously, hSSB1 was reported to function in the HR pathway by stimulating RAD51-mediated D-loop formation (Richard et al., 2008). However, the in vitro CaCl2 conditions required to demonstrate RAD51 stimulation are significantly higher than intracellular levels (Maravall et al., 2000, Ziman et al., 2010, Inami et al., 2013). Surprisingly, we report that, in the presence of reduced CaCl2, both hSSB1 and hSSB2 can independently form D-loop structures in an ATP-independent manner. In our attempts to further characterize the hSSB proteins in HR, we examined the effect of CaCl2 and MgCl2 on the individual activity of hSSB1 and hSSB2 in D-loop formation.

Notably, CaCl2 reduced the activity of hSSB2 to a much lower extent than hSSB1. Conversely, hSSB1 D-loop formation was more sensitive to MgCl2 concentration than hSSB2. The significance of the divalent cations CaCl2 and MgCl2 on the hSSBs activity is not yet known; however, it is possible that the CaCl2 interferes specifically with hSSB2/DNA interaction. Both CaCl2 and MgCl2 bind DNA in the major groove, which may inhibit the hSSBs from binding. Additionally, high levels of CaCl2 (> 5 mM) and, to a lesser extent, MgCl2, stimulate the rate of ssDNA annealing (data not shown). It is possible that the divalent ions interfere with the duplex melting activity of the hSSBs. CaCl2 reduces ATP-hydrolysis by Rad51, which increases recombination activity of Rad51 in vitro while excess MgCl2 deactivates Rad51 activity (Bugreev and Mazin, 2004). However, hSSB1 and hSSB2 do not require ATP to produce
D-loop structures; therefore CaCl₂ and MgCl₂ likely affects the hSSBs using a different mechanism.

Intracellular Ca²⁺ and Mg²⁺ levels vary considerably from the levels used in vitro. Intracellular concentrations of CaCl₂ range between 0.0001 and 0.4 mM, which is significantly lower than the concentrations used in vitro (Maravall et al., 2000, Ziman et al., 2010, Inami et al., 2013). Extracellular Ca²⁺ concentrations, often utilized in cellular signaling, are much higher and range from 2 to 20 mM (Hesketh et al., 1983, Messerli et al., 2007, Celli et al., 2010). Likewise, intracellular Mg²⁺ levels range between 0.1 mM – 0.8 mM while extracellular levels are 0.5 to 20 mM (Westerblad and Allen, 1996, Fox et al., 2007). The concentrations of CaCl₂ utilized in the D-loop assays described here were initially chosen based on previously reported data (Richard et al., 2008) or reduced to near intracellular levels. The MgCl₂ levels in the Rad51-mediated D-loop assay were determined by maximum amount of D-loop product by Rad51 in vitro (data not shown). Going forward, it would be useful to examine the characteristics of HR proteins utilizing physiologically relevant levels of cofactors in vitro.

In addition to characterizing the role of CaCl₂ and MgCl₂ on D-loop formation, I also examined DNA binding by the hSSBs. The DNA binding characteristics of hSSB1 were characterized (Richard et al., 2008, Delagoutte et al., 2011). However, DNA binding activity of hSSB2 was not reported. As expected, hSSB2 preferentially binds ssDNA but not dsDNA. The DNA binding features of the hSSB proteins contributes to the activity of the proteins. For example, the lower affinity of hSSB1 to ssDNA (~10 fold lower compared to RPA) appears to be beneficial, allowing the SSB to stimulate the
activity of several HR enzymes, including the MRN complex and Exo1 (Yang et al., 2012, Delagoutte et al., 2011).

Both hSSB1 and hSSB2 form heterotrimeric complexes with INTS3 and hSSBIP1 (Li et al., 2009, Zhang et al., 2009, Huang et al., 2009, Ren et al., 2014). Individual complex formation of either hSSB1 or hSSB2 with INTS3 and hSSBIP1 has been demonstrated through co-immunoprecipitation, protein affinity purification and crystal formation (Li et al., 2009, Zhang et al., 2009, Huang et al., 2009, Ren et al., 2014). The complex positively impacts the activity of several HR proteins, such as stimulating dsDNA resection activity of Exo1, which is responsible for generating 3’ssDNA tails after DSBs are formed (Yang et al., 2012). Additionally, as a complex, the affinity of hSSB1 for ssDNA is greater than 30-fold higher than hSSB1 alone (Yang et al., 2012). We have purified INTS3 and hSSBIP1 and further supported complex formation of hSSB1, INTS3 and hSSBIP1 through physical interaction in vitro.

Recently, INTS6 was identified in a complex with INTS3 and hSSB1/2 through co-immunoprecipitation (Zhang et al., 2013). However, the function of INTS6 with hSSB1 has not yet been reported. It will be interesting to learn how the new integrator subunit affects hSSB1 individually and as a complex.

References


CHAPTER 4

THE HUMAN MEI5 SUBUNIT OF THE MEI5-SWI5 COMPLEX CONTRIBUTES
MEDIATOR ACTIVITY TO DMC1.

Abstract

Homologous recombination (HR) is responsible for preserving genomic stability and increasing genetic diversity. RAD51 and DMC1, two E. Coli RecA-like recombinases, are essential for HR to occur, and both recombinases utilize several types of enzymes for HR to occur efficiently. Mediators are specifically required by RAD51 and DMC1 to relieve RPA inhibition during HR. In yeast, the Schizosaccharomyces pombe SWI5-SFR1 and Saccharomyces cerevisiae MEI5-SAE3 function as a mediator to DMC1 (Haruta et al., 2006, Ferrari et al., 2009). To date, the human homolog of spSWI5-SFR1, hMEI5-SWI5, has yet to be characterized with hDMC1. In this Chapter, we have purified the human MEI5-SWI5 complex and individual hMEI5 and hSWI5 proteins and demonstrated a physical interaction with both hDMC1 and hRPA. Similar to the yeast orthologs, the hMEI5-SWI5 complex binds both ssDNA and dsDNA, and hMEI5 contributes the DNA binding activity to the complex. Importantly, we have demonstrated that hMEI5 but not hSWI5 overcomes hRPA inhibition of hDMC1-mediated strand exchange.

1. Introduction

Double-strand breaks (DSBs) are the most detrimental type of DNA damage that can occur in cells. If left unrepaired or repaired incorrectly, DSBs can lead to a multitude
of disastrous events, including carcinogenesis, chromosomal aneuploidies or cell death (Nakanishi et al., 2005, Rudin and Haber, 1988, Lim and Hasty, 1996). However, the cell has evolved several mechanisms to repair DSBs, with homologous recombination (HR) as the preferred pathway to accurately repair DSBs and maintain genomic integrity (Haber, 1998, Keeney and Neale, 2006).

HR relies on the action of two RecA-like recombinases, RAD51 and DMC1. RAD51 functions during both mitotic and meiotic HR; however, DMC1 is primarily meiotic specific (Shinohara et al., 1992, Bishop et al., 1992). Both recombinases require mediators such as RAD52 and accessory factors, including RAD54 and RPA, to proceed efficiently (Sung, 1997, Mazin et al., 2003, Haruta et al., 2006). RPA is paradoxical in that it has the ability to both stimulate the rate of RAD51- and DMC1-mediated HR and inhibit recombination by preventing the recombinases from binding ssDNA (Sung, 1997, Sehorn et al., 2004, Haruta et al., 2006). Inhibition occurs when RPA localizes to and binds the ssDNA prior to the recombinases (Sugiyama et al., 1997). Individually, RAD51 or DMC1 are unable to remove RPA and therefore require mediators. Mediators in HR have several common features, including ssDNA binding activity and physical interaction with one or both recombinases (Shinohara et al., 1998, Gasior et al., 2001, Sung et al., 2003). Significantly, mediators also have the ability to overcome RPA-inhibition, allowing the recombinase to proceed with HR activities (Sung et al., 2003).

In the fission yeast *Schizosaccharomyces pombe*, the SWI5-SFR1 complex was shown to function as a mediator to both RAD51 and DMC1 (Haruta et al., 2006, Kurokawa et al., 2008). Alternatively, the budding yeast homolog *Saccharomyces*
*cerevisiae* MEI5-SSA3 retains DMC1-specific mediator activity but does not display RAD51-mediator activity (Ferrari *et al.*, 2009, Say *et al.*, 2011). Recently, two SWI5-SFR1 orthologs in higher eukaryotes (human and mouse) have been identified (Yuan and Chen, 2011, Akamatsu and Jasin, 2010). Both orthologs of hMEI5-SWI5 play a role in HR and functionally interact with RAD51.

The human MEI5-SWI5 complex was implicated in the HR pathway through *in vivo* observation of hMEI5- or hSWI5-depleted cells. In the absence of hMEI5 or hSWI5, the cells displayed decreased RAD51 foci at the site of DSBs, increased sensitivity to ionizing radiation, and reduced HR activity (Yuan and Chen, 2011). Furthermore, both hMEI5 and hSWI5 physically interact with RAD51 *in vitro*, providing compelling evidence for a role of hMEI5-SWI5 in HR (Yuan and Chen, 2011). However, to date, there are no reports regarding hMEI5-SWI5 function with hDMC1. Based on the activity of the yeast MEI5-SWI5 orthologs, we hypothesized that the hMEI5-SWI5 complex likely acts as a mediator to hDMC1.

In this study, we have examined the functionality of hMEI5-SWI5 with hDMC1 *in vitro*. Purified hMEI5, hSWI5, and hMEI5-SWI5 complex physically interact with hDMC1 and hRPA. The DNA binding activity of the hMEI5-SWI5 complex was examined. hMEI5 but not hSWI5 confers the ability to bind both ss and dsDNA. Notably, mediator activity by hMEI5 on DMC1-mediated strand exchange was demonstrated.
2. Materials and Methods

2.1 Protein Purification

2.1a MBP-hMEI5 and MBP-hSWI5 Expression and Purification

The human MEI5 cDNA was inserted into the bacterial expression plasmid pMal-vector (GE Healthcare) to include a maltose-binding protein (MBP) on the N-terminal end of MEI5. The gene was sequenced to ensure no undesired mutations were present. The pMal-MEI5 expression vector was then transformed into E. coli Rosetta (DE3) cells, grown at 37°C to an $A_{600}$ of 0.8 followed by the addition of IPTG to 0.4 mM and incubated for 20 h at 16°C. The cells were harvested by centrifugation using a Beckman JLA16.250 rotor at 4500 g at 4°C. All subsequent steps were carried out at 4°C. 60 g of cell paste was resuspended in 300 mL of Buffer A (50 mM Tris pH 7.5, 1 mM EDTA, 10% sucrose, 0.01% Igepal, 1 mM β-mercaptoethanol, 0.1 mg/mL lysozyme, 0.5 mM benzamidine, 1 mM PMSF, and protease inhibitors: aprotinin, chymostatin, leupeptin, and pepstatin A at a final concentration of 5 µg/mL) containing 150 mM KCl and subjected to sonication at a constant output at of 6 for 30 second cycles 3 times. The extract was clarified by ultracentrifugation in a Beckman Ti.45 rotor at 100,000 g for 90 min at 4°C. The supernatant was diluted 1:1 in Buffer B (20 mM KH$_2$PO$_4$ pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, and 1 mM dithiothreitol) and loaded onto a tandem 45 mL Q and 25 mL SP sepharose column. The flow-through, along with 1 column volume (25 mL) wash with Buffer B containing 75 mM KCl, was incubated with 3 mL Amylose sepharose (GE Healthcare) overnight. The matrix was washed with 30 mL Buffer B containing 1 M KCl, followed by a wash with 30 mL of Buffer B containing
300 mM KCl. The protein was eluted using Buffer B containing 300 mM KCl and 10 mM maltose. Eluted fractions were pooled and diluted 1:3 with Buffer C (20 mM KH$_2$PO$_4$ pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, and 1 mM dithiothreitol) containing 300 mM KH$_2$PO$_4$ with 300 mM KCl before loading onto a 1 mL Macro Hydroxyapatite column (Bio-Rad). The protein was captured in the flow-through and dialyzed with Buffer B containing 300 mM KCl before concentrated to 7.5 mg/mL in a Centricon-30 concentrator (Millipore). Aliquots of the protein were stored at -80°C.

The human SWI5 cDNA was also inserted into a pMal vector, expressed and purified in the same manner as hMEI5 with the exception of the Macro Hydroxyapatite column (Bio-Rad). hSWI5 was dialyzed and concentrated after elution from the amylose matrix. Aliquots of the protein were stored at -80°C.

2.1b hMEI5-SWI5 Complex Expression and Purification

In order to express the hMEI5-SWI5 as a complex, the SWI5 cDNA was amplified through PCR to insert a (HIS)$_6$ tag. After sequence verification, SWI5-(HIS)$_6$ was inserted into the pET-RSF Duet vector (Novagen). Co-transformation of MBP-hMEI5 and hSWI5-(HIS)$_6$ was not successful. Instead, MBP-hMEI5 was transformed into *E. coli* Rosetta (DE3) cells. The pET-RSF-hSWI5-(HIS)$_6$ vector was subsequently transformed into *E. coli* Rosetta (DE3) cells containing MBP-hMEI5. The cells were grown at 30°C to an $A_{600}$ of 0.8 followed by the addition of IPTG to 0.1 mM and incubated for 20 h at 16°C. The cells were harvested by centrifugation using a Beckman JLA 16.250 rotor at 4500 g at 4°C. All subsequent steps were carried out at 4°C. 120 g
of cell paste was resuspended in 600 mL of Buffer A containing 150 mM KCl and subjected to sonication 3 times at a constant output of 4 for 30 second cycles. The extract was clarified by ultracentrifugation in a Beckman Ti.45 rotor at 100,000 g for 90 min at 4°C. The clarified supernatant was incubated with 1 mL Ni-NTA Sepharose 6 Fast Flow beads (GE Healthcare) and 40 mM imidazole overnight at 4°C. The bead slurry was washed with 20 mL Buffer B containing 1M KCl and 40 mM imidazole. The column was then washed with 20 mL of Buffer B containing 300 mM KCl and 40 mM imidazole. The protein was eluted with Buffer B containing 300 mM KCl and 500 mM imidazole. The eluted fractions were incubated with 2 mL Amylose resin (GE Healthcare) overnight. The matrix was washed with 20 mL Buffer B containing 1 M KCl, followed by a wash with 20 mL of Buffer B containing 300 mM KCl. The protein was eluted using Buffer B containing 300 mM KCl and 10 mM maltose. Eluted fractions were pooled and diluted 1:3 in Buffer B containing 100 mM KCl. The diluted fractions were loaded onto a 1 mL Source S column (GE Healthcare). The protein was fractionated with a 30 mL gradient of Buffer C containing 100 mM – 500 mM KCl and peak fractions (~250 mM KCl) were determined by Coomassie staining and pooled before concentrated to 1 mg/mL in a Centricon-30 concentrator. Aliquots of the purified protein were stored at -80°C.

2.1c hDMC1 Purification

The hDMC1 expression plasmid was transformed into E. coli Rosetta (DE3) cells and grown at 37°C to an OD₆₀₀ 0.8 before induced with IPTG to 0.4 mM final. After an additional 16 hr incubation at 16°C, the cells were harvested at 4500 g in a Beckman JLA
16.250 rotor at 4°C. 40 g of cell paste was resuspended in 200 mL of Buffer D (50 mM Tris pH 7.4, 10% sucrose, 150 mM NaSO₄, 0.01% Igepal, 1 mM β-mercaptoethanol, 0.1 mg/mL lysozyme, 1 mM benzamidine, 1 mM PMSF, and protease inhibitors: aprotinin, chymostatin, leupeptin, and pepstatin A at a final concentration of 5 µg/mL) containing 600 mM NaCl and sonicated with 3 times for 30 second cycles. The extract was clarified by ultracentrifugation at 100,000 g for 90 min in a Beckman Ti.45 rotor. The clarified supernatant was diluted 1:4 in Buffer B containing 150 mM KCl and loaded onto a 40 mL Q Sepharose column. After a 120 mL wash with Buffer B containing 150 mM KCl, the bound proteins were fractionated with Buffer B containing 150 mM - 800 mM KCl. After protein composition was analyzed by Coomassie staining, the peak fractions (~ 350 mM KCl) containing hDMC1 were pooled and incubated with 2 mL of Ni-NTA resin (GE Healthcare). The matrix was washed with 20 mL of Buffer B containing 1 M KCl followed by a 20 mL wash with Buffer B containing 300 mM KCl. The bound protein was eluted with 6 mL of Buffer B containing 500 mM imidazole and 300 mM KCl. The eluate was diluted with Buffer B to 100 mM KCl and loaded onto a 1 mL Source S (GE Healthcare). The column was washed with 10 mL of Buffer B containing 100 mM KCl and fractionated with Buffer B containing 100 mM - 400 mM KCl. Peak fractions (~ 150 mM KCl) containing hDMC1 were pooled, diluted with Buffer B to 150 mM KCl and loaded onto a 1 mL Source Q (GE Healthcare). The column was fractionated with Buffer B containing 150 mM - 500 mM KCl. Peak fractions (~ 280 mM KCl) of hDMC1 were determined by Coomassie staining prior to concentration and stored in aliquots at -80°C.
2.1d hRPA Purification

hRPA was purified from *E. coli* Rosetta (DE3) cells following the procedure previously described from Sung, 1997b, with modifications from Sigurdsson *et al.*, 2001. Briefly, 60 g cell paste was resuspended in 300 mL Buffer A containing 150 mM KCl. The sample was subjected to sonication 3 times for 30 second cycles at a constant output at of 6 before ultracentrifugation in a Beckman Ti.45 rotor at 100,000 g for 90 min at 4°C. The clarified supernatant was diluted 1:2 in Buffer B containing 100 mM KCl and loaded onto a 65 mL Q-sepharose column (GE Healthcare). The protein was fractionated in Buffer B with a gradient of 100 mM to 800 mM KCl. Peak fractions were pooled and diluted 1:3 in Buffer C containing 300 mM KH₂PO₄ with 300 mM KCl and loaded onto a 1 mL Macro Hydroxyapatite column (Bio-Rad). The protein was fractionated with a gradient of 0 to 400 mM KH₂PO₄ with 400 mM KCl. Peak fractions were pooled, diluted 1:3 in Buffer C containing 150 mM KCl and loaded onto a 1 mL Source Q (GE Healthcare). Fractions containing RPA were concentrated, and aliquots were stored at -80°C.

2.2 Affinity Pull-down assay

For pull-down experiments using amylose resin, either MBP-MEI5 (2 µg), MBP-SWI5 (2 µg) or MBP-MEI5-SWI5-HIS (2 µg) was first incubated at 4°C for 30 min with either DMC1 (2 µg) in Buffer B containing 100 mM KCl in a final volume of 30 µL. hMEI5 (2 µg) was also incubated with hRPA (2 µg). Amylose resin was then added to the reactions and agitated for 30 min at 4°C. The supernatant (30 µL) was removed from
the resin prior to three 30 µL washes of Buffer B containing 100 mM KCl. Protein bound to the resin was eluted by the addition of 30 µL 2x SDS dye. Equal volumes of 2x SDS dye were added to the supernatant and wash samples. The supernatant, wash and bead elution fractions were subjected to SDS-PAGE analysis on 15% polyacrylamide gels and stained with Coomassie Blue. Additionally, 6 µL of the indicated fraction was loaded into a 15% SDS-PAGE followed by Western analysis using either anti-MBP (Abcam) or anti-HIS (Invitrogen).

2.3 φX174 DNA electrophoretic mobility shift assay

Two plasmid DNA molecules, the φX174 viral (+) strand (ssDNA) and ApaLI digested φX174 replicative form I (dsDNA), were utilized to determine DNA binding in an electrophoretic mobility shift assay. Increasing amounts of MEI5, SWI5 or MEI5-SWI5 complex were incubated at 37°C with either φX174 ssDNA (30 µM nucleotides) or linearized φX174 dsDNA (15 µM base pairs) for 10 min in 12.5 µL of Buffer E (20 mM Tris HCl pH 7.5, 50 mM KCl, 1 mM DTT) containing 100 mM KCl. A control reaction (using the highest concentration for each protein) was deproteinized with Proteinase K (0.5 mg/mL) and SDS (0.5% final) at 37°C for an additional 10 min. The samples were resolved on 1.0% agarose gels and visualized with ethidium bromide. DNA binding activity was analyzed using ImageQuant (GE Healthcare) software.

2.4 Oligonucleotide DNA electrophoretic mobility shift assay

Oligonucleotide H3 was 5'-end labeled with [γ-^32P]-ATP using T4 polynucleotide kinase. Unincorporated [γ-^32P] ATP was removed using Micro Bio-Spin 30 Columns
(Bio-Rad). To construct the 80 bp dsDNA substrate, equimolar amounts of oligonucleotide $^{32}\text{P-OLH3}$ and OLH3-c were incubated at 100°C for 5 min in Buffer F (100 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM EDTA pH 8.0) and slowly cooled to room temperature. The annealed DNA substrates were gel purified on 10% non-denaturing TAE polyacrylamide gels. The substrates were excised from the polyacrylamide gel, electroeluted and filter dialyzed with TE buffer (50 mM Tris, pH 8.0, 1 mM EDTA).

Either $^{32}\text{P-OLH3}$ (ssDNA) or $^{32}\text{P-OLH3} /\text{H3c}$ (dsDNA) (0.05 pmol) was incubated in the presence of increasing concentrations of hMEI5 or hSWI5 for 10 min at 37°C in 10 µL of Buffer F (50 mM Tris–HCl pH 7.5, 1 mM DTT) containing 100 mM KCl. The reaction products were separated on 12% non-denaturing TAE polyacrylamide gels. The gels were dried, analyzed with a phosphorimager and quantified with ImageQuant (GE Healthcare) software. A control reaction was deproteinized by treatment with SDS (0.5% final) and Proteinase K (0.5 mg/mL) at 37°C for 10 min prior to loading on gel.
Table 4.1 Oligonucleotide sequences used in DNA electrophoretic mobility shift assay and homologous pairing assay.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLH3</td>
<td>ssDNA</td>
<td>TTGATAAGAGGTCAATTGAATCCATGGCCTAGAGCT TAATTCGCTGAATCTGGTGCTGGATCCACATGTTT TAAATATG</td>
</tr>
<tr>
<td>OLH3-c</td>
<td>dsDNA</td>
<td>CATATTGAAACATGTGGATCCCAGCACCAGATTC AGCAATTAAGCTCTAAGCCATGAATTCAAATGACC TCTTATCAA</td>
</tr>
<tr>
<td>OL83</td>
<td>ssDNA</td>
<td>AAATGAACATAAAGTAAATAAGTATAAGGATAATA CAATAAGTAAAGTAAATAGAATACAGAAAATAAA GTAAAGGATATAAA</td>
</tr>
<tr>
<td>OL83-c</td>
<td>ssDNA</td>
<td>TTTATATCTCTTTACTTTATTTCTATGTATTTATTCATT TTACCTTTGATTATCCTTATACCTATTATTTAT GTTCATTT</td>
</tr>
</tbody>
</table>

2.5 Homologous DNA pairing assay

To construct the duplex DNA, the 5'-end of OL83 was labeled with $[\gamma^{32}P]$-ATP using T4 polynucleotide kinase. Unincorporated $[\gamma^{32}P]$ ATP was removed using Micro Bio-Spin 30 Columns (Bio-Rad). Annealing of $^{32}P$-OL83 and unlabeled OL83-c was accomplished by heating to 100°C for 5 min in Buffer E before cooling slowly and gel purification from a 10% non-denaturing TAE polyacrylamide gel.

Unlabeled OL83 (10 µM nucleotides) was incubated with 2 µM DMC1 in Buffer G (50 mM Tris pH 7.5, 2.4 mM MgCl$_2$, 2 mM ATP and 1 mM DTT) and an ATP-regenerating system (20 mM creatine phosphatase, 30 µg/mL creatine kinase) at 37°C for 10 min before the addition of MEI5 or SWI5. The reaction was incubated an additional 5 min at 37°C. The duplex DNA (5 µM base pairs), composed of $^{32}P$-OL83 annealed to OL83-c, was added with 1 µL of 50 mM spermidine to the reaction mixture and further
incubated for 120 min at 37°C (final reaction volume 12.5 µL). The reaction was deproteinized by treatment with Proteinase K (0.5 mg/mL) and SDS (0.5% final) at 37°C for 10 min and subjected to 12% native polyacrylamide gel electrophoresis in TAE buffer.

To detect mediator activity, RPA was first incubated with the unlabeled OL83 (10 µM nucleotides) for 5 min at 37°C before either MEI5 or SWI5 was added. After additional 5 min incubation, 2 µM DMC1 was added and the reaction was further incubated for 5 min prior to dsDNA (^{32}P-OL83/OL83-c) being incorporated into the reaction. The remaining steps in the reaction were followed as described for the homologous DNA pairing assay.

3. Results

3.1 Purified hMEI5, hSWI5 and the complex MEI5-SWI5

Both MEI5 and SWI5 were purified using a maltose-binding protein (MBP) to increase solubility (Figure 4.1A, lanes 1-2). Unlike S. cerevisiae SAE3, hSWI5 is insoluble in the absence of an MBP-tag (Say et al., 2011). Therefore, attempts to purify hSWI5-(HIS)_6 alone were unsuccessful. Co-expression of MBP-hMEI5 and hSWI5-(HIS)_6 slightly increased solubility and allowed purification of the complex (Figure 4.1A, lane 3). The MEI5-SWI5 complex remained stable after a 1M KCl buffer wash and co-eluted from the Source S column, indicating that hMEI5-SWI5 forms a stable complex (data not shown). Western blot analysis using either anti-MBP or anti-HIS (as indicated) was utilized to confirm the presence of each protein (Figure 4.1B, lanes 1-3).
Figure 4.1 Purified hMEI5, hSWI5 and MEI5-SWI5. (A.) Purified MBP-hMEI5 (lane 1), MBP-hSWI5 (lane 2), and MBP-hMEI5-hSWI5-HIS complex (lane 3). (B.) Western analysis of purified proteins using anti-MBP (panel I) or anti-HIS antibodies (panel II).

3.2 hMEI5, hSWI5 and the complex MEI5-SWI5 physically interact with hDMC1 and hRPA

In yeast, multiple methodologies have demonstrated an interaction between MEI5-SAE3 and DMC1, including co-localization on recombination hotspots and physical interaction (Tsubouchi and Roeder, 2004, Ferrari et al., 2009). Additionally, previous studies have shown a direct interaction between MEI5-SAE3 and RPA in yeast (Ferrari et al., 2009). We also observed a direct physical interaction of hMEI5, hSWI5, and hMEI5-SWI5 with hDMC1 using an affinity pull-down assay (Figure 4.2A, lanes 1-3, 4-6 and 7-9, respectively). The MEI5-SWI5 complex has a slightly higher affinity for DMC1 compared to MEI5 or SWI5 individually. hMEI5, hSWI5, and hMEI5-SWI5 (in the complex, only hMEI5 had a MBP-tag) bound the amylose beads through the MBP-tag
and were found in the elution (Figure 4.2A, lanes 3, 6, and 9). Purified hDMC1 does not have an MBP-tag; therefore, any DMC1 found in the elution fractions was a result of interaction with MEI5, SWI5, or hMEI5-SWI5. As expected, hDMC1 did not bind the amylose beads in the absence of an interacting partner (Figure 4.2A, lanes 10-12). A Western analysis using anti-HIS was utilized to confirm the presence of hDMC1 in the elution fractions (Figure 4.2A, lower panel). Additionally, hMEI5 independently interacted with RPA (Figure 4.2B, lanes 1-3). RPA did not interact with the amylose beads independently (Figure 4.2B, lanes 4-6).

![Figure 4.2 hMEI5, hSWI5 and MEI5-SWI5 complex physical interaction with hDMC1 and hMEI5 interaction with hRPA.](image)

Figure 4.2 hMEI5, hSWI5 and MEI5-SWI5 complex physical interaction with hDMC1 and hMEI5 interaction with hRPA. (A.) hMEI5 (lanes 1-3), hSWI5 (lanes 4-6) and MEI5-SWI5 complex (lanes 7-9) were incubated with DMC1 before the addition of Amylose resin to capture the MBP-tagged proteins. DMC1 was incubated alone with the Amylose resin (lanes 10-12). (B.) hMEI5 was incubated with RPA before addition of Amylose resin (lanes 1-3). The supernatant (S), wash (W) and elution (E) were analyzed after electrophoresis on a 15% SDS-PAGE and stained with Coomassie Brilliant Blue. Lanes 4-6 were a control with RPA and amylose.
### 3.3 hMEI5 contributes DNA binding activity to the MEI5-SWI5 complex

*S. cerevisiae* MEI5-SAE3 has been reported to possess DNA binding activity using plasmid-length DNA substrates (Say et al., 2011). However, the mouse homolog SWI5-SFR1 apparently does not retain DNA binding activity (Akatmatsu and Jasin, 2010). To determine if hMEI5, hSWI5 or the complex possesses DNA binding activity, a DNA electrophoretic mobility shift assay (EMSA) with plasmid-length substrates, ϕX174 ssDNA and linearized ϕX174 RF dsDNA, was performed. Increasing concentrations of hMEI5, hSWI5 or the complex was incubated with either ssDNA or dsDNA. MBP-hMEI5 shifted both ssDNA (Figure 4.3A, lanes 2-5) and dsDNA (Figure 4.3B, lanes 2-5). MBP-hSWI5 was unable to bind either ssDNA (Figure 4.3A, lanes 8-11) or dsDNA (Figure 4.3B, lanes 8-11). Like MEI5, the hMEI5-SWI5 complex shifted both ssDNA (Figure 4.3A, lanes 14-17) and dsDNA (Figure 4.3B, lanes 14-17), with a slight preference for ssDNA (Figure 4.3A and B, lane 16). These results indicate the hMEI5-SWI5 complex is capable of binding both ssDNA and dsDNA, similar to the activity displayed by the yeast homolog. Additionally, MEI5 is responsible for the DNA binding activity of the MEI5-SWI5 complex.
Figure 4.3 hMEI5 and hMEI5-SWI5 but not hSWI5 can bind ϕX174 DNA. (A.) Electrophoretic mobility shift assay (EMSA) using ssDNA. The indicated concentrations of hMEI5 (I), hSWI5 (II), or hMEI5-SWI5 complex (III) were incubated with ϕX174 ssDNA (30 µM nucleotides) at 37°C for 10 min. The reaction products were separated on 1.0% agarose gels and stained with ethidium bromide. The gels were quantified using ImageQuant (GE Healthcare) software and graphed. Lanes 1, 7 and 13 contained no protein (NP). Lanes 6, 12 and 18 were deproteinized by treatment with Proteinase K (PK) (0.5 mg/mL) and SDS (0.5% final) at 37°C for 10 min prior to gel electrophoresis. (B.) EMSA using dsDNA. Increasing concentrations (as indicated) of hMEI5 (I), hSWI5 (II), or hMEI5-SWI5 complex (III) were incubated with linearized ϕX174 RF dsDNA (30 µM base pairs). All other steps remained the same as in A.
3.4 hMEI5 and the complex bind both single- and double-stranded oligonucleotides.

Plasmid-length ssDNA has the potential to form secondary structure, including dsDNA (Benevides et al., 1991). To examine whether hMEI5, hSWI5 or the hMEI5-SWI5 complex exhibited any substrate specificity, we utilized 80-bp $^{32}$P-labeled oligonucleotides designed to minimize secondary structure to construct ssDNA ($^{32}$P-OLH3) or dsDNA ($^{32}$P-OLH3/OLH3c) substrates. Similar to the results seen with the plasmid-length φX174 DNA, both hMEI5 (Figure 4.4A and B, panel I) and the hMEI5-SWI5 complex (Figure 4.4A and B, panel III) were able to bind both ssDNA and dsDNA, with little difference in affinity. It appears that both hMEI5 and the hMEI5-SWI5 complex may have a slightly higher binding preference for ssDNA compared to dsDNA (Figure 4.4A and B, lanes 5 and 16). hSWI5 was again unable to bind either DNA substrate (Figure 4.4A and B, panel II).
Figure 4.4 hMEI5 confers the DNA binding activity to the hMEI5-SWI5 complex. (A.) Both hMEI5 and the hMEI5-SWI5 complex bind ssDNA. Increasing concentrations (as indicated) of hMEI5 (Panel I), hSWI5 (Panel II) or hMEI5-SWI5 (Panel III) were incubated with 0.05 pmol [32P]-OLH3 (ssDNA) for 10 min at 37°C before separation on a native polyacrylamide gel. A control lane (All panels lane 6) was deproteinized by treatment with Proteinase K (0.5 mg/mL) and SDS (0.5% final) at 37°C for 10 min prior to electrophoresis, designated by S/P. Lanes 1, 7 and 13 contained no protein (NP). The gels were quantified using ImageQuant software and graphed. hSWI5 did not bind the ssDNA at the concentrations used, indicating that hMEI5 contributes DNA binding activity to the complex. (B.) Both hMEI5 and hMEI5-SWI5 also bind dsDNA. Increasing concentrations (as indicated) of hMEI5 (Panel I), hSWI5 (Panel II) or hMEI5-SWI5 (Panel III) were incubated with 0.05 pmol [32P]-OLH3/OLH3c (dsDNA) for 10 min at 37°C. All other steps were followed as previously described in (A).
3.5 Human MEI5 and hSWI5 do not stimulate hDMC1 strand exchange.

To test the effect of MEI5 and SWI5 on DMC1-mediated strand exchange in vitro, we utilized a homologous DNA pairing assay. Briefly, DMC1 forms a filament on the ssDNA before the addition of the complementary $^{32}$P-labeled dsDNA oligonucleotide. Strand exchange occurs when the recombinase exchanges the complementary unlabeled ssDNA with the $^{32}$P-labeled strand of the dsDNA duplex (Figure 4.5 Panel A). The products are detected based on the migration pattern after electrophoresis (ssDNA vs dsDNA).

At the indicated concentration, hDMC1 alone can catalyze about 25% strand exchange (Figure 4.5A Panel II, lane 2). Increasing concentration of either hMEI5 (Figure 4.5A Panel II, lanes 3-7) or hSWI5 (Figure 4.5A Panel II, lanes 8-12) did not significantly alter DMC1-mediated strand exchange. As expected, strand exchange did not occur in the absence of DMC1 (Figure 4.5A Panel II, lane 1).
Figure 4.5 hMEI5 and hSWI5 effect on hDMC1-mediated strand exchange activity. (A.) hMEI5 or hSWI5 does not stimulate hDMC1 strand exchange. Panel I provides a schematic of the homologous DNA pairing assay. hDMC1 (2 µM) was incubated with unlabeled OL83 at 37°C for 5 min before the addition of hMEI5 or hSWI5 (at the indicated concentrations) for 5 min. The reaction was initiated by the addition of $^{32}$P-OL83/OL83-c and further incubated for 120 min. The reactions were deproteinized and separated on 12% native polyacrylamide gel electrophoresis. Lane 1 is a control with no protein. hDMC1 was incubated alone (Panel I lane 2) or in the presence of increasing MEI5 (Panel I lanes 3-8) or hSWI5 (Panel I lanes 9-12). The reaction products were quantified and graphed in panel III.

3.6 Human MEI5 but not hSWI5 overcomes RPA inhibition and functions as a mediator to DMC1.

The homologous DNA pairing assay was also utilized to determine mediator activity by hMEI5 and hSWI5. When added prior to DMC1, RPA prevented DMC1 strand exchange activity (Figure 4.6A Panel II, lanes 2-3). However, hMEI5 was able to alleviate RPA inhibition and restore DMC1-mediated strand exchange (Figure 4.6A Panel II, lanes 4-8). At the highest concentration, MEI5 can restore approximately 50% of DMC1 activity (Figure 4.6A Panel III, lanes 2 and 8). Conversely, hSWI5 had no
effect on RPA inhibition and was unable to restore DMC1 strand exchange (Figure 4.6A Panel II, lanes 9-13).

4. Discussion

Homologous recombination (HR) is the most accurate DSB repair mechanism and is critical to the survival of a cell (Krogh and Symington, 2004). Therefore, identifying proteins that regulate the recombinases in HR provides important mechanistic details and
generates possible targets for cancer therapies. In this paper, we have provided the first report on the effect of hMEI5-SWI5 on DMC1 recombination activity in vitro.

Strand exchange is an integral part of the HR pathway and occurs when the recombinase forms a nucleoprotein complex on the invading and donor DNA molecule (Bianco et al., 1998). Numerous HR proteins, such as Rad52 and HOP2-MND1, have been shown to stimulate either RAD51 or DMC1 during the strand exchange process (Krejci et al., 2002, Chi et al., 2007, Pezza et al., 2007). Furthermore, the mouse ortholog SWI5-SFR1 stabilizes RAD51-filament formation, increasing the rate of RAD51 strand exchange (Tsai et al., 2012). We hypothesized that hMEI5 or hSWI5 may also stimulate DMC1-mediated strand exchange activity. However, neither hMEI5 nor hSWI5 stimulated Dmc1 strand exchange activity under the conditions used here.

Mediators are required to remove RPA from ssDNA and assist the recombinase in loading onto the free ssDNA, allowing strand exchange to proceed (Sung, 1997, Haruta et al., 2006). MEI5-SAE3 has been identified as a mediator to DMC1 in Saccharomyces cerevisiae through the ability to overcome RPA inhibition (Ferrari et al., 2009). Additionally, SWI5-SFR1, the MEI5-SAE3 homolog in Schizosaccharomyces pombe, functions as a mediator to both Rhp51 (RAD51 homolog) and DMC1 (Haruta et al., 2006, Murayama et al., 2013).

Based on the physical interaction of hMEI5-SWI5 with both DMC1 and RPA and previous reports of mediator activity in the homologs, we decided to test hMEI5 and hSWI5 for mediator activity. Here, we demonstrated that hMEI5 but not hSWI5 acts as a mediator to hDMC1 by alleviating RPA-inhibition and restoring DMC1-mediated strand exchange.
exchange activity. However, the mechanism in which hMEI5 mediates is not yet known. Mediators, such as hMEI5-SWI5, alleviate RPA inhibition and promote recombinase-mediated HR. hMEI5 interacts with RPA (Fig. 4.2B, lane 3) and binds ssDNA (Fig 4.3A Panel 1 and Fig 4.4A Panel 1). It is possible that hMEI5 utilizes ssDNA binding activity while binding to RPA to overcome inhibition (Figure 4.6). DMC1-mediated strand exchange activity was partially restored after hMEI5 stimulated the release of RPA from the ssDNA.

**Figure 4.7** MEI5-SWI5 relieves RPA inhibition on DMC1-mediated homologous recombination. In response to DNA DSBs, RPA (green) is rapidly recruited to and tightly binds ssDNA. The hMEI5-SWI5 complex (blue and yellow) interacts with RPA in addition to ssDNA, perhaps stimulating RPA to release the ssDNA molecule. MEI5-SWI5 may also bind the exposed ssDNA to prevent RPA from re-binding before DMC1 (gray) is assisted in filament formation during pre-synapsis. Filament formation proceeds through synapsis after relief from RPA inhibition.
Due to the difficult nature (unstable proteins and nuclease-prone) and low yield (as low as 50 µg total from 60 g of cell paste) of the hMEI5-SWI5 complex purification, I have been unable to purify sufficient hMEI5-SWI5 complex to examine the effect of the complex on hDMC1-mediated strand exchange or hRPA inhibition. Given the similarities between hMEI5 and the hMEI5-SWI5 complex described in this report, it is likely that the hMEI5-SWI5 complex will, in the least, be able to restore hDMC1 activity comparable to hMEI5. Under that assumption, the effect of the hMEI5-SWI5 complex on hDMC1-mediated strand exchange and hRPA inhibition would provide compelling support to the hMEI5 mediator activity shown here. Instead, I have characterized the DNA binding activity of the complex in addition to establishing a physical interaction with hDMC1.

Similar to the yeast ortholog MEI5-SAE3, the hMEI5-SWI5 complex also binds both ssDNA and dsDNA (Say et al., 2011). Surprisingly, the mSWI5-SFR1 does not have the ability to bind either ssDNA or dsDNA (Tsai et al., 2012). The lack of DNA binding activity by the mSWI5-SFR1 may be related to the 16 aa RSfp motif (rodent SFR1 proline rich motif) located in the N-terminal domain of MEI5 (Akamatsu and Jasin, 2010). Although the RSfp motif is found in mammals, the motif is repeated to varying degrees in rodents only and appears to function as a negative regulator of SWI5-SFR1 (Akamatsu and Jasin, 2010).

Despite the lack of DNA binding activity, the mSWI5-SFR1 was shown to stimulate RAD51 strand exchange activity (Akamatsu and Jasin, 2010). Specifically, mSWI5-SFR51 stabilizes the RAD51 pre-synaptic filament formation, effectively

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increasing the rate of RAD51-mediated HR (Tsai et al., 2012). *In vitro*, hMEI5-SWI5 physically interacts with hRAD51 (Yuan and Chen, 2011). Additionally, it appears the hMEI5-SWI5 complex contributes to hRAD51 localization to the site of DNA DSBs. Human cell lines depleted of either MEI5 or SWI5 displayed significantly reduced RAD51 foci formation after exposure to ionizing radiation (Yuan and Chen, 2011). Further studies aimed towards the mechanism of hMEI5-SWI5 mediator activity to hDMC1 would be beneficial.

The fission yeast ortholog, *Schizosaccharomyces pombe* SWI5-SFR1, also stimulates spRHP51 strand exchange activity, and recent studies have identified one interesting mechanism in which spSWI5-SFR1 might stimulate strand exchange (Tsai et al., 2012). The spSWI5-SFR1 appears to stimulate strand exchange by perpendicularly aligning DNA bases to the RAD51-filament axis, increasing the organization and stimulating activity (Fornander et al., 2014). It is possible that increased organization of the DNA bases would likely facilitate a search for homology in the invading duplex DNA molecule (Fornander et al., 2014). Notably, a DNA-binding mutant of spSWI5-SFR1 increased the rate of RAD51 strand exchange, albeit at a higher concentration than needed for the wild type, indicating that direct interaction of spSWI5-SFR1 with the DNA was irrelevant (Fornander et al., 2014). It is possible that the mouse SWI5-SFR1 complex functions through a similar mechanism as spSWI5-SFR1.

There are several similarities between the yeast MEI5 homologs, SFR1 and human MEI5. For example, SFR1 or MEI5 is the contributing protein to the DNA binding activity for both yeast and human complexes (Ferrari et al., 2009). Both human
and yeast MEI5, SWI5/SAE3, and both complexes all physically interact with DMC1, and importantly, both the yeast MEI5-SAE3 and human MEI5 have the ability to remove RPA from ssDNA to promote DMC1-mediated strand exchange (Ferrari et al., 2009). Although differences are found in each of the MEI5-SWI5 orthologs in yeast as well as mouse SWI5-SFR1, a common function is mediator activity. Future work needs to be focused on the hMEI5-SWI5 complex and the mechanism at which hMEI5 utilizes to overcome RPA inhibition with hDMC1.

References


CHAPTER 5

SUMMARY

Homologous recombination (HR) is the preferred pathway to repair DNA double-strand breaks (DSBs). If repaired incorrectly, DSBs are detrimental to the survival of a cell (Rudin and Haber, 1988, Carney et al., 1998, Lim and Hasty, 1996). In response to DSBs, transducer kinases are activated to initiate a phosphorylation signaling cascade, leading to cell cycle arrest and nucleolytic resection to generate ssDNA tails (Petrini, 2000, Kastan and Lim, 2000). In eukaryotes, RAD51 and DMC1 are the only RecA like recombinases and are critical for HR to occur.

There are three phases in the HR pathway: pre-synapsis, synapsis, and post-synapsis. Pre-synapsis begins when the recombinase forms a nucleoprotein filament on the 3’ ssDNA tail, stretching the DNA molecule in a search for homology with a duplex donor strand (Sung and Robberson, 1995, Conway et al., 2004). Once found, a displacement loop (D-loop) is formed in the dsDNA, beginning synapsis (Bianco et al., 1998). The recombinase forms a synaptic complex between the donor and invading ssDNA molecule to facilitate ATP-dependent strand exchange (Bianco et al., 1998, Chi et al., 2006). Utilizing ATP hydrolysis, accessory proteins dissociate the recombinase from the duplex DNA during post-synapsis (Solinger et al., 2002, Kiianitsa et al., 2006). Following DNA replication by polymerases, the duplex molecules are resolved using one of the following pathways: synthesis-dependent strand annealing (SDSA), break-induced replication (BIR) or double-strand break repair (DSBR).
Cross-over formation is prevented in SDSA by disruption of the D-loop structure, and therefore the SDSA pathway is predominately utilized in mitotic cells (Kadyk and Hartwell, 1992, Barber et al., 2008, Cromie et al., 2006). BIR requires extensive leading and lagging strand DNA synthesis, which can lead to loss of heterozygosity and is consequently employed when only one end of the DSB is available (Bosco and Haber, 1998). In DSBR, post-synapsis is an intricate process, where the resolution of a double Holliday junction leads to either a non-crossover or crossover event (Gilbertson and Stahl 1996, Wu and Hickson, 2003, Cejka and Kowalczykowski, 2010, Shi et al., 2009). DSBR is preferred during meiosis, where cross-over formation is beneficial (Keeney and Neale, 2006).

Efficient HR is accomplished by the action of mediators and accessory proteins on the recombinases. Mediators can be defined by three characteristics: (1) the ability to bind ssDNA, (2) physical interaction with a recombinase and (3) ability to overcome Replication protein A (RPA) inhibition (Shinohara et al., 1998, Gasior et al., 2001, Sung et al., 2003). RPA has a high affinity for ssDNA and localizes rapidly to DSBs, preventing RAD51- and DMC1- nucleoprotein complex formation on the ssDNA (Yang et al., 2013, Sugiyama et al., 1997, Haruta et al., 2006, Sung, 1997). Mediators can either remove RPA through physical interaction or assist the recombinase with loading onto free ssDNA (Sung et al., 2003).
**Human Mei5 and Swi5**

Several homologs of the human MEI5-SWI5 complex are mediators to the recombinases, such as *S. pombe* SWI5-SFR1 or to only one recombinase (Haruta *et al.*, 2006, Kurokawa *et al.*, 2008). *S. cerevisiae* MEI5-SAE3 only has mediator activity to scDMC1 (Ferrari *et al.*, 2009, Say *et al.*, 2011). Currently, hMEI5-SWI5 appears to function in the HR, based on *in vivo* observations and a physical interaction with hRAD51 (Yuan and Chen, 2011). We have provided further evidence to support the function of hMEI5-SWI5 in HR. We have successfully purified MBP-hMEI5 and MBP-hSWI5 individually. However, the hMEI5-SWI5 complex proved to be much harder to purify. Through sustained efforts, I was able to purify the hMEI5-SWI5 complex, albeit at a very low yield.

Based on the activity of the MEI5-SWI5 homologs, we tested hMEI5-SWI5 for characteristics of a mediator. First, we demonstrated a physical interaction with the recombinase hDMC1. Indeed, hMEI5, hSWI5 and hMEI5-SWI5 all interact DMC1. We also demonstrated a physical interaction between hMEI5 and hRPA. Both hMEI5 and hMEI5-SWI5 have the ability to bind both ssDNA and dsDNA. However, hSWI5 does not bind DNA. We incorporated both plasmid-length molecules and oligonucleotides in electrophoretic mobility shift assays to observe DNA binding.

Importantly, we examined the individual proteins, hMEI5 and hSWI5, for stimulation of hDMC1-mediated HR and mediator activity. Although we did not observe any stimulation on hDMC1 strand exchange, hMEI5 did act as a mediator to hDMC1, by overcoming hRPA inhibition and thus, promoting hDMC1 strand exchange. hSWI5 was
not able to stimulate hDMC1 or overcome hRPA inhibition. Therefore, the role of hSWI5 in the MEI5-SWI5 complex is not yet known. hSWI5 physically interacts with hDMC1 but does not bind either ssDNA or dsDNA. It may be that hSWI5 stimulates other activities of hDMC1 such as ATP hydrolysis or filament stability. Alternatively, hSWI5 perhaps mediates hRAD51 HR activity. Further work characterizing the effect of hSWI5 on DMC1 and RAD51 would be useful in determining the role of hSWI5 in HR.

**Future Directions of hMEI5 and hSWI5**

Going forward, it would be useful to purify a sufficient quantity of the hMEI5-SWI5 complex, as only the individual proteins were tested for mediator activity. Protein expression, cell breakage conditions and purification steps have previously been optimized. To increase the quantity of purified protein, I would suggest at least three cell breakage steps (60 g each) and suspending the purification after elution from Ni-NTA resin. All three Ni-NTA elutions should be combined before continuing with the purification protocol. Additionally, hMEI5-SWI5 activity on hRAD51 should be further characterized biochemically.

**Human Single-strand DNA Binding Proteins**

In eukaryotes, there are at least three single-strand DNA binding (SSB) proteins: RPA, hSSB1 and hSSB2. RPA functions in and is required during DNA replication, repair and recombination (Wang et al., 2005, Sung, 1997, Wold, 1997). hSSB1 and hSSB2 have been shown to function in the HR pathway of DNA repair (Richard et al., 2008, Feldhahn et al., 2012, Shi et al., 2013). Recent genetic analysis has indicated the
hSSBs also have a role in the repair of stalled replication forks (Feldhahn et al., 2012, Bolderson et al., 2014).

hSSB1 was first implicated and characterized in the HR by Richard et al. (2008). Specifically, hSSB1 was shown to significantly stimulate RAD51-mediated D-loop formation in the presence of 5 mM CaCl$_2$. We also tested hSSB2 in RAD51-mediated D-loop formation and confirmed the stimulatory effect of hSSB1. However, we also observed hSSB1 and hSSB2 D-loop formation, independent of RAD51. To further characterize hSSB D-loop formation, we completed a series of CaCl$_2$ and MgCl$_2$ titrations and report that CaCl$_2$ has a more profound inhibition effect on hSSB2 D-loop formation while MgCl$_2$ slightly reduced hSSB1 activity. Finally, we purified the hSSB complex proteins, INTS3 and hSSBIP1 and confirmed interaction between the complex proteins.

In our biochemical characterization of the hSSBs, we discovered a novel activity of hSSB1 and hSSB2. Both SSBs can anneal ssDNA and have the ability to melt duplex DNA. Surprisingly, the hSSBs also independently form D-loops in an ATP-independent manner. These activities led us to hypothesize that hSSB1 and hSSB2 may functionally interact with HR polymerases. We demonstrated a physical interaction between the hSSBs and human Polymerase η, and convincingly demonstrated the ability of Pol η to synthesize DNA from hSSB-formed D-loops. In agreement with recently published reports, the work presented here supports the role of the hSSBs in the restart of stalled replication forks. Both hSSB1 and hSSB2 could maintain genomic stability through stimulation hPol η activity on stalled replication forks (Fig 5.1).
Figure 5.1 hSSB1 and hSSB2 may assist hPol η in the restart of stalled replication forks. Stalled replication forks occur during normal replication due to topological stress or DNA damage. hPol η is recruited to the replication fork after stalling. hSSB1 and hSSB2 may assist hPol η and stimulate DNA extension. The role of INTS3 and hSSBIP1 in the repair of replication forks is still unknown.

Future Directions of hSSB1 and hSSB2

Future work involving the hSSBs should look deeper into the functional interactions with other recombination polymerases, including Pol δ. The effect of INTS3 and hSSBIP1 on hSSB D-loop activity should be characterized, and importantly, the role of the hSSB1/2 complex on polymerase activity would be very interesting to examine. It is likely that, along with INTS3 and hSSBIP1, the stimulation of polymerase activity seen
by the hSSBs will proceed more efficiently than in the presence of hSSB1 or hSSB2 individually.

References


APPENDICES
APPENDIX A

SUPPLEMENTARY INFORMATION FOR CHAPTER 2
Figure A.1 Maximum likelihood phylogenetic tree. Constructed from multiple sequence alignment of single strand binding proteins (SSBs). The phylogeny includes representatives from the three domains of life. Different taxonomic groups were identified by colors (Bacteria: green, Archaea: red, Eukaryota: blue). Reference sequences: *Escherichia coli* SSB (P0AGE0): golden, *Sulfolobus solfataricus* (AAK42515): magenta and *Homo sapiens* SSB1 (Q9BQ15): black and *Homo sapiens* SSB2 (Q96AH0): cyan, have a unique color for differential identification and localization in the phylogeny. The outgroup, *Homo sapiens* Replication factor (P35244), is colored gray.
INTS3 STIMULATES HUMAN POLYMERASE ETA ACTIVITY ON D-LOOP STRUCTURES

The human single-strand DNA binding (SSB) proteins function in homologous recombination (HR) and the restart of stalled replication forks (Richard et al., 2008, Bolderson et al., 2014). In HR, the hSSBs have been shown to play a role in Rad51 localization and stimulation of DNA resection after DNA double-strand breaks (DSBs) (Richard et al., 2008, Li et al., 2009, Skaar et al., 2009). Both hSSB1 and hSSB2 form an independent heterotrimeric complex with INTS3 and hSSBIP1, termed sensor of single-stranded (SOSS) DNA (Huang et al., 2009, Li et al., 2009). INTS3 plays an important role in the SOSS complex, serving as a scaffold for hSSB1, hSSB2 and hSSBIP1 localization (Skaar et al., 2009). However, the role INTS3 and hSSBIP1 on hpol η activity has not been determined.

In Chapter 2, I demonstrated hPol η extension from an oligonucleotide D-loop structure was stimulated by hSSB1 and hSSB2. Here, I show that INTS3 also stimulates hPol η. Notably, INTS3 significantly stimulates hPol η activity approximately 30% higher than either hSSB1 or hSSB2. hSSBIP1 slightly increased hPol η D-loop extension; however, the increase in activity was only observed after the last time point taken.
Figure B.1 INTS3 but not hSSBiP1 stimulates DNA synthesis of hPol η from an oligonucleotide D-loop structure. (A.) hPol η (0.08 µM) was incubated either alone (lanes 1-4, panel I) or in the presence of 0.27 µM hSSB1 (lanes 5-8, panel I), 0.18 µM hSSB2 (lanes 9-12) 0.12 µM INTS3 (lanes 13-16) or 0.48 µM hSSBiP1 (lanes 17-20) for 30 min at 37°C in 50 mM Tris-HCl pH 7.4, 1 mM DTT, 4 mM MgCl₂, 0.125 mM dATP, dCTP, dGTP, dTTP each. Reaction products were deproteinized by treatment with SDS and Proteinase K before electrophoresis on an 8% native polyacrylamide gel. Markers on the left indicate the possible combinations formed during annealing. Markers on the right include the ³²P-labeled overhang substrate and the final extended D-loop product. (B.) The percentage of extended D-loop product (marker on the right) was quantified using ImageQuant.
Stimulation of hPol η by INTS3 is intriguing and needs to be further examined. It will be interesting to determine whether the SOSS complex together can stimulate polymerase activity even greater than is observed individually. Although notable stimulation of polymerase activity by hSSBIP1 was observed, it is possible that hSSBIP1 may contribute to the complex activity. Taken together, the SOSS complex plays an important role in the repair of damaged DNA during both HR and replication.

References


APPENDIX C

HSSB1 AND HSSB2 STIMULATE HUMAN POLYMERASE DELTA EXTENSION FROM D-LOOP STRUCTURES

Human polymerase δ functions in homologous recombination and replicates the lagging strand during DNA replication (Maloisel et al., 2008, Li et al., 2013, Wilson et al., 2013). Previous data has suggested the proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) are required for detectable DNA synthesis by hpol δ in vitro (Li et al., 2009, Overmeer et al., 2010). In chapter 2, I demonstrated polymerase η stimulation from a D-loop formed by the human single-strand DNA binding (hSSB) proteins, indicating a role for the hSSBs in the repair of stalled replication forks. As demonstrated with hPol η, it is possible hPol δ may also functionally interact with the hSSBs.

Both hSSB1 and hSSB2 can independently form D-loop structures (Fig. C.1 Panel II, lanes 2 and 6). Importantly, hPol δ extended the D-loop structure formed by both hSSB1 and hSSB2 (Fig. C.1 Panel II, lanes 3-5 and 7-9). hPol δ appears to replicate slightly faster on hSSB2-formed D-loops; however, additional studies are needed to confirm this observation.
Here, I have demonstrated that both hSSB1 and hSSB2 can stimulate DNA synthesis by hPol δ on D-loop structures. Further work is needed to confirm the activity of the hSSBs and to examine the effect of INTS3 and hSSBIP1 individually and as a complex with the hSSBs. Importantly, DNA extension by hPol δ was demonstrated in
the absence of PCNA and RFC. Interaction between the hSSBs with PCNA or RFC would be interesting to characterize. Interestingly, hPol δ stalls on telomere replication forks, and hSSB1 has recently been implicated in the repair of damaged telomere DNA (Lormand et al., 2013, Gu et al., 2013). The data presented here and the co-localization of hPol δ and hSSB1 on telomeric DNA suggests the hSSBs may stimulate polymerases during all stages of DNA repair (replication, DSB repair and telomeric DNA).

References


